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THE ACTION OF MONOALKYL AND DIALKYL SUCCINIC ACIDS AND THE MONOESTERS AND MONOAMIDO-DERIVATIVES OF ALKYL SUCCINIC ACIDS ON THE GROWTH OF *MYCOBACTERIUM TUBERCULOSIS*.

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Received for publication March 7, 1947.

THE following paper records the results of experimental work undertaken to find new compounds which would inhibit the growth of *Mycobacterium tuberculosis*.

EXPERIMENTAL.

Methods.

Various compounds were tested against *Myco. smegmatis*, and if they prevented or delayed the growth of this organism they were tested against *Myco. tuberculosis*. The medium used was nutrient peptone digest broth which, when it was desired, was enriched with 5 per cent pooled human serum, and the chemicals tested were subsequently added. The organisms used were *Myco. smegmatis* and *Myco. tuberculosis* (bovis) No. 46; both were obtained from the National Collection of Type Cultures. After repeated subculturing these grew well in the above medium. The inocula used were made as uniform as possible by making the bacterial suspensions as even as possible and bringing to a standard density; each inoculum of *Myco. tuberculosis* weighed *ca.* 0·005 mg. Final readings of the growth of *Myco. smegmatis* were taken on the 6th day and of *Myco. tuberculosis* on the 42nd day of incubation, and compared with the controls. The chemicals were also tested for haemolytic action on standard suspensions of washed human red blood cells at 37° C. for one hour.

Results.

The work of Shriner and Adams (1932) on the action *in vitro* of branched chain fatty acids on the growth of acid-fast bacteria, and of Robinson and Birch (1940) on the action of trisubstituted acetic acids, have aroused interest in branched chain fatty acids as possible anti-tubercular compounds. Many different substances were tested for an anti-bacterial action *in vitro* against the acid-fast bacilli without success until the late Professor T. J. Nolan (1937) suggested to Dr. V. Barry, of University College, Dublin, that roccellic acid, a product isolated from the lichen *Lecanora sordida* and having a branched chain structure, might

be inhibitory to the growth of the mycobacteria. This acid is a disubstituted succinic acid (α-methyl-α^1-*n*-dodecylsuccinic acid) having the structure:

$$\begin{matrix}CH_3\\COOH\end{matrix}>CH.CH<\begin{matrix}C_{12}H_{25}\\COOH\end{matrix}$$

This acid, however, proved to be weakly inhibitory. It prevented the growth of *Myco. smegmatis* in a dilution of 1/1000, but not at 1/2000. It prevented the growth of *Myco. tuberculosis* (bovis) in a dilution of 1/2000 and delayed growth at 1/4000, but had no effect at 1/5000. It was also haemolytic at 1/1000 against human, rabbit and guinea-pig washed red blood cells. Similar results were obtained with other dialkylated succinic acids, such as α-methyl-α^1-*n*-hexadecylsuccinic acid and $\alpha\alpha^1$-di-*n*-heptylsuccinic acid. When, however, an ethyl alcoholic solution of the anhydride of any of the above acids was neutralized with a dilute alkali by Dr. Barry only one equivalent of the alkali was taken up, and the neutral solution contained a mixture of the isomeric monoethyl esters, i.e.:

$$\begin{matrix}R_1\\COOH\end{matrix}>CH.CH<\begin{matrix}R_2\\COOC_2H_5\end{matrix}, \text{ and } \begin{matrix}R_1\\COOC_2H_5\end{matrix}>CH.CH<\begin{matrix}R_2\\COOH\end{matrix}$$

Solutions prepared in this way gave very interesting and hopeful results on testing, and lead to the synthesis and development of a large number of new chemical compounds. The methods used in the synthesis of these compounds are described elsewhere by Barry and Twomey (1947).

Tables I, II and III summarize the results of *in vitro* experiments with these compounds. The most effective action occurred when R_1 of the half esters of the

TABLE I.—*Antibacterial Action of the Monoethyl Esters of Monoalkyl Succinic Acids.*

Acid.	R_1.	Biological activity.		
		Sm.	M.tbc.	Haem. index.
n-Hexyl	6	1,000	1,000	300
n-Heptyl	7	2,000	3,000	500
n-Octyl	8	3,000	25,000	2,000
n-Nonyl	9	4,000	20,000	5,000
n-Decyl	10	10,000	25,000	10,000
n-Undecyl	11	15,000	50,000	20,000
n-Dodecyl	12	20,000	50,000	20,000
n-Tridecyl	13	70,000	300,000	100,000
n-Tetradecyl	14	90,000	400,000	100,000
n-Pentadecyl	15	70,000	200,000	100,000
n-Hexadecyl	16	30,000	200,000	100,000
n-Octadecyl	18	10,000	50,000	20,000

R_1 = Number of carbon atoms in the alkyl substituent.

Sm. = Dilution of the sodium salt of the half ester which causes complete inhibition of growth of *Myco. smegmatis* for 6 days.

M.tbc. = Dilution of the sodium salt of the half ester which causes complete inhibition of growth of *Myco. tuberculosis* (bovis) for 42 days.

Haem. index = Dilution of the half ester which produces haemolysis in a suspension of washed human red blood cells.

TABLE II.—*Antibacterial Action of the Monoethyl Esters of the Dialkyl Succinic Acids.*

Acid.	$R_1 + R_2$.	Biological activity.		
		Sm.	M.tbc.	Haem. index.
α-Butyl-α¹-hexyl	10	10,000	10,000	2,000
α-Amyl-α¹-hexyl	11	10,000	25,000	10,000
αα¹-Dihexyl	12	10,000	75,000	20,000
α-Isoamyl-α¹-octyl	13	20,000	200,000	50,000
α-Methyl-α¹-dodecyl	13	40,000	400,000	100,000
α-Propyl-α¹-decyl	13	30,000	200,000	50,000
α-Ethyl-α¹-dodecyl	14	60,000	300,000	200,000
αα¹-Diheptyl	14	60,000	400,000	100,000
αα¹-Dioctyl	16	20,000	100,000	100,000
α-Methyl-α¹-hexadecyl	17	20,000	50,000	50,000

$R_1 + R_2$ = Total number of carbon atoms in both substituents.
Other column headings as in Table I.

TABLE III.—*Antibacterial Action of Monomethyl Esters of Monoalkyl and Dialkyl Succinic Acids.*

Acid.	R_1 or $R_1 + R_2$.	Biological activity.		
		Sm.	M.tbc.	Haem. index.
αα¹-Dihexyl	12	5,000	25,000	50,000
α-Methyl-α¹-dodecyl	13	40,000	300,000	200,000
α-Ethyl-α¹-dodecyl	14	20,000	200,000	200,000
αα¹-Diheptyl	14	10,000	100,000	100,000
n-Hexadecyl	16	10,000	75,000	100,000
αα¹-Dioctyl	16	10,000	75,000	100,000

Column headings as in Tables I and II.

TABLE IV.—*Antibacterial Action of Various Half Esters of α-Methyl-α¹-n-dodecyl-succinic Acid (Roccellic Acid).*

Half ester.	Biological activity.		
	Sm.	M.tbc.	Haem. index.
Monomethyl	40,000	500,000	50,000
Monoethyl	40,000	400,000	50,000
Monoallyl	20,000	400,000	50,000
Monopropyl	20,000	400,000	50,000
Monobutyl	40,000	300,000	100,000
Monoamyl	20,000	300,000	50,000
Monocyclohexyl	20,000	300,000	50,000
Monoglycerol	10,000	100,000	50,000

Sm. = Dilution of the half ester which causes complete inhibition of growth of *Myco. smegmatis* for 6 days.
M.tbc. = Dilution of the half ester which causes complete inhibition of growth of *Myco. tuberculosis* (bovis) for 42 days.
Haem. index = Dilution of the half ester which produces haemolysis in a suspension of washed human red blood cells.

monoalkyl succinic acids, and $R_1 + R_2$ of the half esters of the dialkyl succinic acids, contained 13 or 14 carbon atoms. There was comparatively little difference in effectiveness of action between the derivatives of the monoalkyl and dialkyl preparations provided R_1 and $R_1 + R_2$ equalled the same number of carbon atoms.

Half esters of α-methyl-α¹-*n*-dodecylsuccinic acid (roccellic acid) were prepared employing a number of different alcohols:

$$\begin{matrix}CH_3\\COOR\end{matrix}>CH.CH<\begin{matrix}C_{12}H_{25}\\COOH\end{matrix}, \text{ and } \begin{matrix}C_{12}H_{25}\\COOR\end{matrix}>CH.CH<\begin{matrix}CH_3\\COOH\end{matrix}$$

Tests with these compounds (Table IV) showed that no increase in the *in vitro* activity was produced by altering the molecule in this way.

Various monoamido-derivatives of α-methyl-α¹-*n*-dodecylsuccinic acid (roccellic acid) were next prepared, and also produced a mixture of isomers:

$$\begin{matrix}CH_3\\CONHR\end{matrix}>CH.CH<\begin{matrix}C_{12}H_{25}\\COOH\end{matrix}, \text{ and } \begin{matrix}CH_3\\COOH\end{matrix}>CH.CH<\begin{matrix}C_{12}H_{25}\\CONHR\end{matrix}$$

The biological tests (Table V) with these derivatives showed no improvement in inhibition on our previous results and, apart from the *p*-aminobenzenesulphonamide compound, were disappointing.

TABLE V.—*Antibacterial Action of Various Half Amido-derivatives of Roccellic Acid.*

Amido-derivative.	Biological activity.		
	Sm.	M.tbc.	Haem. index.
Half amido	5,000	100,000	5,000
Half anilide	10,000	200,000	20,000
Half toluidide	5,000	50,000	20,000
Half piperidide	5,000	10,000	20,000
p-Aminobenzoic ester compound	5,000	100,000	20,000
p-Aminophenol compound	10,000	75,000	20,000
α-Napthylamine compound	5,000	25,000	10,000
p-Chloraniline compound	10,000	50,000	100,000
p-Aminobenzenesulphonamide compound	5,000	400,000	50,000
2-Aminopyridine compound	20,000	100,000	20,000
2-Aminothiazole compound	10,000	100,000	100,000
2-*p*-aminobenzenesulphonamide-pyridine compound	10,000	75,000	20,000

Column heading abbreviations as in Table I.

The inhibitory action of these compounds against *Myco. tuberculosis* (bovis) No. 46 was not confined to this strain alone: the monoethyl ester of di-*n*-heptylsuccinic acid (at a dilution of 1/300,000) caused complete inhibition of the growth of *Myco. tuberculosis* (bovis) No. 14, and of another very virulent bovine strain which had recently been isolated; at the same dilution is also completely inhibited the growth of *Myco. tuberculosis* H 37 R V (Trudeau) for 42 days.

Effect of serum on anti-bacterial activity.

It was found that pooled human serum when added to the medium partially inhibited the action of the esters and even reduced their effectiveness *in vitro* five or ten times. Whole blood, citrated blood and plasma also had this effect. In a series of experiments the albumin, globulin and " protein-free " fractions of human serum were separated as far as possible by using saturated ammonium sulphate, 30 per cent trichloracetic acid, heating, centrifuging and dialysis, and then bringing back to the original volume with normal saline. The different fractions and normal human serum were put up in diminishing amounts (1/10, 1/20, 1/40, 1/80, 1/100, 1/320, 1/640) with 1/30,000 of the monomethyl ester of α-methyl-α^1-*n*-dodecylsuccinic acid in the culture medium; the test organism was *Myco. tuberculosis* (bovis). It was found that normal human serum at a concentration of 1/40 gave full protection against anti-bacterial activity, but gave no protection at a concentration of 1/80. The albumin fraction gave protection at 1/20, but the globulin and " protein-free" fractions gave no protection at 1/10. The antagonism between the anti-bacterial substance and the serum is thus due mainly to the albumin fraction. It is possible that the serum albumin forms a stoichiometric complex with the antibacterial substance, but probably an important factor is the growth-promoting effect of serum albumin *in vitro* on *Myco. tuberculosis* (Boissevain, 1940).

Effect of serum on haemolytic activity.

The addition of normal human serum (1/10) raised the concentration of these compounds necessary for haemolysis by, on the average, about a hundred times; and a series of haemolytic experiments using decreasing amounts of human serum (1/10 to 1/128) and increasing amounts of the sulphapyridine derivative of

TABLE VI.—*Inhibition of the Haemolytic Activity of a Roccellic Acid Derivative by Serum and Serum Fractions.*

The haemolytic agent was 0·20 ml. 1/4000 tyrosine-ethyl derivative of roccellic acid in 2·0 ml.

Dilution of—	Whole serum.	Fraction of serum.		
		Albumin.	Globulin.	" Protein-free."
1/4	−	−	+	−
1/8	−	−	+	−
1/16	−	−	+	+
1/20	−	−	+	+
1/24	−	−	+	+
1/32	−	−	+	+
1/40	−	−	+	+
1/48	−	+	+	+
1/64	−	+	+	+
1/80	+	+	+	+
1/96	+	+	+	+
1/128	+	+	+	+
Control (no serum)	+	+	+	+
Control (no ester)	−	−	−	−

+ = haemolysis; − = No haemolysis.

α-methyl-α¹-*n*-dodecylsuccinic acid showed that the haemolytic action of this compound was proportional to the amount of serum used.

Another series of haemolytic experiments were undertaken, using different fractions of human serum in varying amounts against the tyrosine ethyl derivative of α-methyl-α¹-*n*-dodecylsuccinic acid in the presence of washed human red blood cells. The serum fractions were separated as before. The results (Table VI) confirmed the findings of the protective action of serum albumin on bacterial growth, and suggest that the formation of the stoichiometric complex is an important factor in this action.

Activity against other organisms.

Staphylococcus aureus.—Varying dilutions of different esters were put up against this organism in ordinary laboratory nutrient broth, the inoculum being $1{\cdot}1 \times 10^5$ cells/c.c. The presence or absence of bacteria and the number of organisms present were determined after 24 hr. The results (Table VII) confirmed those obtained with the mycobacteria and showed that 13 or 14 carbon atoms in the substituents (R_1 or $R_1 + R_2$) gave the more effective compounds, and that *Staph. aureus* was susceptible to their action.

TABLE VII.—*Effect of Succinic Acid Derivatives on the Growth of* Staph. aureus.

Monoethyl ester of—	Dilution of ester (1 g. in *x* ml.) which	
	Prevented growth.	Lessened growth.
n-Decylsuccinic acid (C_{10})	10,000	17,000
αα¹-Dihexylsuccinic acid (C_{12})	12,500	33,000
n-Dodecylsuccinic acid (C_{12})	17,000	80,000
α-Methyl-α¹-dodecylsuccinic acid (C_{13})	20,000	80,000
α-Propyl-α¹-decylsuccinic acid (C_{13})	50,000	100,000
αα¹-Diheptylsuccinic acid (C_{14})	60,000	80,000
αα¹-Dioctylsuccinic acid (C_{16})	25,000	80,000
α-Methyl-α¹-hexadecylsuccinic acid (C_{17})	2,000	12,500
n-Octadecylsuccinic acid (C_{18})	2,000	8,000

C. diphtheriae (mitis).—The inoculum used was 5×10^4 cells/c.c. The monoethyl ester of α-methyl-α¹-*n*-dodecylsuccinic acid inhibited the growth of this organism in glucose broth at 1/40,000, and with serum (5 per cent) added to the medium at 1/10,000. Growth occurred in glucose broth in the presence of the half-ester in a dilution of 1/80,000.

Esch. coli and Aerobacter aerogenes.—Normal growth took place in 24 hr. with a dilution of 1/1000 of the monoethyl ester of α-methyl-α¹-*n*-dodecylsuccinic acid in the medium.

Bacteriostatic or bactericidal?

A broth culture of *Staph. aureus* (24 hr.) was diluted 1/1000, and 1 c.c. inoculated into 9 c.c. broth containing 1/5000 of the monoethyl ester of α-methyl-α¹-*n*-dodecylsuccinic acid and incubated at 37° C. Bacterial counts were done at hourly intervals and the results (given in Table VIII) showed a definite bactericidal action; the growth between the third and fourth hours is of interest, since

the organisms enter an active growth phase and become more susceptible to the bactericidal effect.

TABLE VIII.—*Bactericidal Action of Roccellic Acid Monoethyl Ester on* Staph. aureus.

Time (hr.).	Bacteria/ml.	Time (hr.).	Bacteria/ml.
0	72,000	8	10
1	1,600	9	0
2	400	10	0
3	60	11	0
4	360	12	0
5	25	14	0
6	15	24	0
7	10	48	0

SUMMARY.

1. A new series of compounds has been found which markedly inhibit the growth of *Myco. tuberculosis.*

2. The substances are strongly haemolytic against red blood cells.

3. The anti-bacterial and haemolytic actions are most marked when the combined substituents contain 13 or 14 carbon atoms.

4. The anti-bacterial and haemolytic actions are reduced by serum albumin.

5. The compounds are strongly antibacterial against *Staphylococcus aureus* and *C. diphtheriae,* but not against *E. coli* or *Aerobacter aerogenes.*

6. The anti-bacterial action is bactericidal.

My thanks are due to Prof. R. A. Q. O'Meara for many fruitful discussions, and to Dr. V. Barry for the synthesis and supply of the compounds. My thanks are also due to the Medical Research Council of Ireland, who financed the investigation, and under whose direction the work is being carried out.

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THE STABILIZATION OF THROMBIN IN PLASMA: DEVELOPMENT OF A SIMPLE TWO-STAGE METHOD FOR THE DETERMINATION OF PROTHROMBIN.

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ALTHOUGH one-stage methods for the determination of prothrombin (Quick, 1935, 1939; Quick, Stanley-Brown and Bancroft, 1935; Shapiro, Sherwin, Redish and Campbell, 1942) have gained increasing popularity during recent years, it is nevertheless recognized that the ideal procedure (Quick, 1942) would involve a quantitative conversion of prothrombin to thrombin, and determination of the latter by its ability to clot a standard solution of fibrinogen. The chief difficulty in the application (Warner, Brinkhous and Smith, 1936; Smith, Warner and Brinkhous, 1937) of this principle has been the progressive inactivation of thrombin by antithrombic substances immediately after its formation. In the present work a procedure has been developed for the suppression of the antithrombic activity of plasma. The necessity of working—as hitherto—with highly diluted plasma is thus obviated. Furthermore, the variable introduced by the time interval between the first stage of conversion of prothrombin to thrombin and the second stage of measurement of thrombic activity by fibrinogen is excluded. Plasma is defibrinated by thrombin just as in the method of Warner *et al.*, but the suppression of the antithrombic activity of plasma makes it possible to produce thrombin by a very simple procedure so that the method can be applied in any clinical laboratory.

RESULTS.

A. *Stabilization of thrombin by alcohol.*

Assuming that the reaction of thrombin with antithrombic substances is of a non-specific nature, possibly adsorption, we tried various substances that might interfere with this reaction and found that alcohol possesses such a property. For ethanol a 25 per cent (but not 17 per cent) concentration proved satisfactory.

(Wherever in this paper a certain concentration of alcohol is mentioned, this relates to concentration by volume to the volume of the total liquid in which the alcohol is contained.) At 25 per cent concentration not only is the antithrombic activity of plasma suppressed, but the aggregation of fibrin to form a real clot is also prevented, although there appears a coarse granularity, if an ethyl alcohol plasma is allowed to clot at this concentration with calcium and thromboplastin.

This granularity is due to fibrin, as it does not appear if thrombin is produced in a defibrinated alcohol plasma upon addition of calcium and thromboplastin.

Ethanol at a concentration of 25 per cent was more satisfactory in the suppression of antithrombic activity than higher alcohols used in concentrations

corresponding to that of 25 per cent ethanol according to Traube's rule (Langmuir, 1917).

The suppression of antithrombic activity by alcohol is a reversible reaction. If alcohol plasma, to which calcium and thromboplastin has been added (" thrombinized " alcohol plasma), is diluted with normal saline solution, its thrombic activity disappears rapidly.

If plasma, stabilized by 25 per cent alcohol concentration and " thrombinized " with calcium and thromboplastin, is added to an equal volume of fibrinogen solution or fresh plasma (thereby reducing the alcohol concentration to one half) a clot is produced. This clot forms irrespective of whether the thrombin containing plasma is defibrinated before thrombinization (clear solution) or not. The clotting is due to a specific action of thrombin upon fibrinogen, as it does not take place if decalcified alcohol plasma—with or without addition of excess thromboplastin—is added to a solution of fibrinogen.

B. *Effect of varying the time of addition of alcohol to plasma clotting* (a) *with and* (b) *without the addition of excess of thromboplastin.*

Plasma was allowed to clot with calcium, in the first series with, and in the second without, the addition of excess thromboplastin. Sufficient ethyl alcohol to form a solution of 25 per cent was added at various intervals. The " thrombinized " plasmas were tested for thrombic activity with fresh plasma (fibrinogen). The results are given in Tables I and II.

The effectiveness of alcohol stabilization is shown in Table I. No change in thrombic activity occurred during the first three hours after stabilization, and deterioration was relatively slight during a subsequent four-hour period. Thrombic activity was greatest when alcohol was added at the start of the clotting reaction (minute 0), and became progressively less when alcohol was added later. This shows that in the absence of alcohol, antithrombic activity is very marked; but as soon as alcohol was added, the thrombic activity of such plasma (serum) stabilized only after clotting remained constant for a long time.

TABLE I.—*Plasma " Thrombinized " with Addition of Excess Thromboplastin.*

(1·6 c.c. plasma + 0·2 c.c. normal saline extract of acetone dehydrated rabbit brain + 0·2 c.c. of 0·2 M calcium chloride).

Time of test (minutes after addition of alcohol).	Time of addition of alcohol. Clotting times (seconds).					
	0	1	2	4	6	10 minutes.
8	5·0	7·8	17·4	22·0	23·1	31·4
15	4·9	8·0	14·6	22·9	23·2	36·0
30	5·6	7·6	11·0	17·0	22·2	31·0
66	4·3	6·5	13·5	23·3	20·3	25·7
120	5·6	7·5	10·3	22·9	22·2	40·2
180	6·0	7·8	9·8	21·8	34·0	51·5
240	17·2	9·5	11·3	25·3	30·8	64·3
420	18·8	13·8	21·3	25·1	36·6	more than 90

(All clots were left in the solution during the entire test.)

TABLE II.—*Plasma "Thrombinized" without Addition of Thromboplastin.*

(1·6 c.c. plasma + 0·2 c.c. normal saline solution + 0·2 c.c. of 0·2 M calcium chloride).

Time of test (minutes after addition of alcohol).	Time of addition of alcohol. Clotting times (seconds).					
	0	1	2	4	6	10 minutes.
8	92·5	21·0	19·9	12·2	6·5	33·6
15	113·2	20·0	18·5	10·0	14·5	30·6
30	77·2	12·7	40·0	21·6	17·7	32·4
66	97·8	17·5	12·3	26·8	12·4	24·2
120	38·2	7·0	6·8	7·1	9·9	31·0
240	14·9	6·8	6·0	6·0	9·0	38·8
420	7·6	5·9	5·9	5·4	9·4	35·9

Clotting of the original plasma occurred six minutes after the addition of calcium (room temperature). It coincided visibly with the addition of alcohol in the case of the six minutes' column. Clots were left in the solutions in the cases of the last two columns.

In the plasmas allowed to clot without addition of excess thromboplastin (Table II) thrombin concentrations immediately after stabilization were smaller than in Table I. They increased, however, with time of incubation with alcohol, although it took 7 hours to reach the 5 to 7 seconds level, while without addition of alcohol this activity was produced in 6 minutes (first row in the 6 minutes' column). This shows that alcohol impairs the conversion rate of prothrombin to thrombin; but the liberation of new thrombin from prothrombin after 7 hours' incubation with alcohol demonstrates that alcohol has no large destructive effect upon prothrombin, and probably does not decrease the final yield of thrombin.

The fact that the thrombin-(fibrinogen) plasma clotting times became longer when alcohol was added with increasing delay to plasma clotted with addition of excess of thromboplastin (Table I), and that these increased clotting times do not become shorter again on standing, shows that alcohol is unable to recover thrombin already inactivated by antithrombic substances.

Inactivation of stabilized thrombin becomes apparent earlier in the series with excess of thromboplastin than in those without addition of thromboplastin seemingly because of the lack of continued formation of thrombin in the former.

c. *Determination of prothrombin.*

I. *Principle.—step one:* Oxalated plasma is defibrinated with thrombin, and the clot removed after 15 minutes.

Step two: The defibrinated plasma is stabilized by the addition of alcohol and "thrombinized" by human milk (thromboplastin) (Jacoby and Adler, 1937; Kraszewski and Lindenfeld, 1935; Solé, 1935; Freudenberg, 1944) and calcium.

Step three: Dilutions of the "thrombinized" plasma are set up.

Step four: The thrombic activity of various dilutions of the "thrombinized" stabilized plasma is measured by adding 0·2 c.c. to an equal volume of fresh normal oxalated human plasma and recording the dilution in which clotting appears in 15 seconds.

II. *Reagents.*—25 per cent ethyl alcohol in normal saline solution.
50 per cent ethyl alcohol in normal saline solution.
0·1 M sodium oxalate solution.
0·2 M calcium chloride solution.
Thrombin solution.
Fresh normal human oxalate plasma.
Human milk.

Preparation of thrombin stock solution: principle.—Whole blood is stabilized by addition to alcohol, and the prothrombin converted to thrombin by addition of excess thromboplastin (human milk).

Preparation of thrombin stock solution, procedure.—Place in a series of test tubes 5·0 c.c. of 50 per cent alcohol in normal saline and 2·5 c.c. of normal saline solution. Draw blood by venepuncture and place into each test tube 2·5 c.c. Shake by inverting the tube three times. Add immediately to each test tube 1·0 c.c. of human milk and 1·0 c.c. of 50 per cent alcohol in normal saline. Shake again by inverting the tube six times. (Do not mix the milk with the 50 per cent alcohol solution beforehand.) The material in the test tubes will become gelatinous after some time. It should be shaken violently before use to break up the gelatinous (fibrin-like) mass. If 0·2 c.c. of this solution is added to 0·2 c.c. of fresh human plasma, clotting will occur in 6 to 7 seconds. This thrombin solution is remarkably stable, keeping at room temperature for at least one month. It should not be stored in the ice box.

Preparation of thrombin for defibrination.—A thrombin stock solution that has been allowed to stand at room temperature for three days or longer should be used for this procedure. (No fresh solution should be employed.) At this time a precipitate will have formed in the thrombin stock solution. Therefore, shake well before use to obtain a uniform suspension. To prepare about 5 c.c. of "defibrination thrombin" place 10 c.c. of such thrombin suspension into a centrifuge tube and centrifugalize for 10 minutes at about 1200 r.p.m. Decant the alcohol-containing supernatant. Add to the sediment 5 c.c. of normal saline solution, shake well to suspend all the sediment, and centrifugalize again at low speed for about 10 minutes. Pipette off the supernatant and use it as defibrination thrombin. Antithrombic activity has been largely suppressed by using old rather than fresh thrombin stock solution for the preparation of "defibrination thrombin." (Reasons for using saline, rather than alcoholic thrombin solutions, for defibrination will be given below.)

Fresh human plasma.—9 c.c. of blood are drawn by venepuncture from a normal subject with as little trauma as possible, and added immediately to 1 c.c. of 0·1 M sodium oxalate solution contained in a centrifuge tube. Shake by inversion of the tube three times and obtain the plasma by centrifugalization.

III. *Procedure.*—4·5 c.c. of blood is drawn by venepuncture as rapidly, and with as little trauma as possible, and added to a centrifuge tube containing 0·5 c.c. of 0·1 M sodium oxalate solution. Shake by inverting the tube three times. Obtain the plasma by centrifugalization.

Step one: Defibrination.—To 1·0 c.c. plasma add 1·0 c.c. defibrination thrombin freshly prepared from an old thrombin stock solution. (It is possible, but not desirable, to leave the defibrination thrombin at room temperature for about two hours before using it.) Ensure good mixing before clotting has started. After 15 minutes remove the clot by wrapping it around a glass rod and thereby

expressing the defibrinated plasma. The thrombic activity of the thrombin added has been neutralized by this time by antithrombic substances.

Step two : Thrombinization.—To 1·0 c.c. of defibrinated plasma add successively :

1·25 c.c. of 25 per cent alcohol in normal saline solution +
1·375 c.c. of 50 per cent alcohol in normal saline solution +
0·3 c.c. of human milk +
0·075 c.c. of 0·2 M calcium chloride solution,
shaking after each addition.

A 1/8 dilution of the original plasma has thus been obtained.

Step three : Dilution.—Serial dilutions of such thrombinized plasma should be set up in the time interval of 10 minutes to 1 hour after thrombinization. The clotting tests should be done during this period.

For dilution a 25 per cent alcohol solution in normal saline is used. We usually set up dilutions of 40, 20, 10 and 5 per cent of thrombinized plasma (corresponding to 5, 2·5, 1·25 and 0·625 per cent of the original plasma).

Step four : Reading.—With a serological blowing pipette 0·2 c.c. of various dilutions of " thrombinized " plasma are added to Wassermann tubes containing 0·2 c.c. of fresh human plasma. At the moment of contact with the plasma a stop-watch is started. The test tube is held after brief shaking against a screened source of light (an electric bulb screened by placing filter-paper in front of it proved satisfactory). The test tube is tilted in a way that the fluid contained in it is allowed to flow in turn along its walls from the bottom of the tube towards its top, and back to the bottom again, and the moment of appearance of granularity is recorded by stopping the stop-watch. In high dilutions some cloudiness becomes apparent before the formation of a real granularity, but the appearance of the latter is taken as the end point. End points are very sharp in this procedure. The whole test is done at room temperature.

D. *Application of the method.*

The present method does not require controls and results could be expressed in units. However, the method requires that a certain volume of thrombin is added to an equal volume of fresh plasma when testing for thrombin clotting times. If less plasma is used, no clotting occurs because the alcohol concentration is not reduced sufficiently. Therefore, should we attempt to give our results in units, we should have to define a new unit differing from those hitherto in use (Smith *et al.*, 1937 ; Warner *et al.*, 1936), in the definition of which other ratios of thrombin to fibrinogen were employed. In order to abstain from introducing new units, we prefer to give our results in percentage, taking 100 per cent as normal.

Table III gives the clotting times of stabilized thrombin produced from an undiluted normal plasma, and from the same plasma after it has been diluted to 50, 30, 20, and 10 per cent with 0·85 per cent saline solution. 1·0 c.c. of each dilution of plasma was treated like an unknown ; it was defibrinated with 1·0 c.c. defibrination thrombin, and " thrombinized " by adding alcohol, human milk and calcium to produce 4·0 c.c. of thrombinized plasma (1/8 dilution of the original plasma). Serial dilutions were set up subsequently and clotted with fresh (fibrinogen) plasma.

TABLE III.—*Amount of Thrombin Obtained from Undiluted and Diluted Normal Plasma.*

Dilution of "thrombinized" plasma (corresponding to 1/8th of original plasma).	Dilution of original plasma (each dilution treated like an unknown.) Clotting times (seconds)				
	100%.	50%.	30%.	20%.	10%.
100 per cent .	..	..	..	13·4	19·0
80 ,, .	..	..	13·0	14·4	20·2
60 ,, .	7·2	9·7	14·8	16·4	24·3
40 ,, .	8·8	12·1	16·3	19·6	..
20 ,, .	13·3	16·3	31·2		
10 ,, .	16·8	21·3			
5 ,, .	20·6				

It will be seen by comparison of the clotting times that the amount of thrombin recovered corresponds to the amount of prothrombin present in the original plasma tested.

Table IV gives the clotting times for various dilutions of thrombinized stabilized plasma obtained in 61 determinations on normal subjects.

TABLE IV.—*Normal Clotting Times of Thrombinized Stabilized Plasma.*

Dilution of thrombinized plasma (per cent) .	100	80	60	40	20	10	5
Corresponding dilution of original plasma (per cent) . . .	12·5	10·0	7·5	5	2·5	1·25	0·625
Number of cases (N) .	43	25	30	54	61	61	60
Mean clotting time (M seconds) . . .	6·2	6·9	7·8	9·2	12·6	18·0	24·7
Standard error of mean .	0·09	0·14	0·15	0·14	0·13	0·17	0·29
Standard deviation (σ) .	0·60	0·73	0·77	1·1	1·0	1·45	2·2
Lower limit of normal ($M - 2\sigma$) . . .	5·0	5·5	6·3	7·0	10·6	15·3	20·3
Upper limit of normal ($M + 2\sigma$) . . .	7·4	8·3	9·3	11·4	14·6	20·7	29·1

It will be seen from Table IV that the normal thrombin clotting time of 15 seconds occurs at approximately 15 per cent concentration of thrombinized plasma, which corresponds to about 2 per cent concentration of the original plasma. Should the clotting time of 15 seconds (14 to 16 seconds) lie between 20 and 40 per cent concentration of thrombinized plasma, a 50 per cent prothrombin concentration would result. If the value of 14 to 16 seconds does not lie between two concentrations examined, but rather coincides with one of them, say 20 per cent concentration, the significance of this can be confirmed by comparing the clotting times of the other concentrations examined with the normal clotting times given for these concentrations in Table IV. If a correponding lengthening of clotting times is observed for these other concentrations, significance is given to the deviation of the clotting time of 15 seconds from 15 to 20 per cent, and a prothrombin level of 75 per cent is recorded.

TABLE V.—*Comparison of Quick's and Author's Method for the Determination of Prothrombin.*

Case.	Prothrombin concentration (per cent). Quick's method.	Author's method.
61 bloods taken from 33 normal subjects at various time intervals during a period of one month	Controls (taken as 100 per cent)	100
Thrombo-embolic disease :—		
E. C—, post-operative saphenous vein thrombosis :		
January 20	100	150
,, 26	170	150
G. A—, post-operative pulmonary infarction :		
January 26	140	200
February 5	..	150
,, 11	170	150
,, 16	170	150
C. H—, post-operative pulmonary infarction	100	150
B. A—, prolapse of uterus, history of recurrent thrombophlebitis. Before operation (Jan. 31).	140	150
After operation (no thrombosis took place)—		
February 9	140	100
,, 11	140	100
,, 13	..	150
,, 16	140	200
Dicumarol treatment—		
L. A. St—, normal subject before dicumarol (Feb. 1)	100	100
During dicumarol administration—		
February 5	..	75
,, 9	50	75
,, 10	50	50
,, 11	50	50
,, 12	35	35
S. C—, post-operative thrombophlebitis, pulmonary infarction, before dicumarol—		
February 5	..	150
During dicumarol administration—		
February 10	50	75
,, 11	40	50
,, 12	25	35
* ,, 13	25	35
E. M—, severe infectious hepatitis during pregnancy	10	10
G. O—, hypoproteinemia of obscure origin :		
January 20	100	100
February 12	75	75

* While this paper was in press, the method described here has been used successfully as a routine procedure for the control of the clinical administration of dicumarol.

Table V gives the results in percentage of comparative determinations of prothrombin by the method of Quick and by the present method upon—

(*a*) Normal subjects.
(*b*) Patients with thrombo-embolic disease.
(*c*) Subjects treated with dicumarol.
(*d*) A patient with severe jaundice during pregnancy, and
(*e*) A patient with hypoproteinemia of obscure origin.

Remarks upon defibrination.—In early experiments we used for defibrination an alcoholic solution of thrombin (prepared as described for the thrombin stock solution above). However, it soon became evident that with this procedure low results are obtained, particularly if the plasma tested is hypoprothrombinemic. This effect became more marked with increasing amounts of thrombin used for defibrination. It was not due to the alcohol contained in it. The effect could be practically eliminated by introducing saline solutions of thrombin for defibrination instead of the alcoholic solution originally employed.

It has been stated that purified thrombin inactivates its precursor, prothrombin (Mertz, Seegers and Smith, 1939). But Ferguson (1941) believes that this is not due to thrombin itself, but to an independent proteolytic factor. Our observation shows that a crude alcoholic thrombin solution inactivates prothrombin, but is in agreement with the opinion of Ferguson that this effect is not due to thrombin itself.

Remarks on the thromboplastin used.—Human milk proved to be a satisfactory thromboplastin for this work. It has the advantage of easy accessibility, and can be applied without special preparation before its use. We usually employed fresh human milk, but a sample kept in the refrigerator for 14 days proved satisfactory in one series, while in another two series, three-day-old milks were used.

Remarks on the fibrinogen plasma.—No variations in the results were observed when different normal human plasmas were used as fibrinogen for the determination of thrombin. Dilution of the fibrinogen plasma caused significant changes only if it exceeded 50 per cent. We have no experience with the use of pathological plasmas.

DISCUSSION.

In comparison with the Iowa two-stage method for the determination of prothrombin (Warner *et al.*, 1936; Smith *et al.*, 1937) the present method has the advantage of simplicity. The reagents are easy to prepare, so that the method can be performed in any clinical laboratory. In the two-stage method of Warner *et al.* the antithrombic activity is minimized by dilution of the "thrombinized" plasma. The objection has been raised (Quick, 1942) that the diminution of antithrombic activity is in itself a variable, in that the dilution required for hypoprothrombinemic plasma is much smaller than that required for normal plasma to give a thrombin concentration which will clot a fibrinogen solution in 15 seconds. The method described above excludes the variable introduced by antithrombic activity.

In comparison with the method of Quick (Quick, 1935, 1939; Quick *et al.*, 1935) the present method has the advantage that the result is obtained in absolute values, and no comparison with a relatively large number (Aggeler, Howard, Lucia, Clark and Astaff, 1946) of normal controls is necessary. The method

does not depend upon the activity of reagents employed, and thromboplastins of relatively low activity (human milk) are satisfactory for "thrombinization" of plasma. A further advantage of the present method is its performance at room temperature. If Quick's method is used for hypoprothrombinemic plasmas the clotting time of undiluted plasma is compared with that of a diluted normal standard. In the standard not only is the prothrombin diluted, but also fibrinogen and other possible clotting factors are affected. Only if fibrinogen is more than 30 per cent of normal is there no significant effect upon the prothrombin clotting time (Witts, 1942). In a diluted standard of normal total plasma the dilution of fibrinogen tends, therefore, to increase the clotting time. This tends to give false high values of prothrombin for the unknown. The factor of inaccuracy introduced by fibrinogen has been eliminated in modifications of Quick's method (Deutsch and Gerarde, 1946; Thordarson, 1940). On the other hand, factors inhibiting the conversion rate of prothrombin to thrombin continue to be postulated (Ferguson and Glatzko, 1941; Tocantins, 1946). If the prothrombin concentration is specifically reduced, other things remaining constant, these inhibiting factors will be relatively more effective. In the diluted normal standards used for comparison in Quick's test inhibition factors also become reduced, and therefore, shorter clotting times are expected than would occur had the concentration of these factors been kept constant. For an unknown—in which prothrombin is reduced and inhibition factors are normal—comparison with such a standard would tend to give false low values of the unknown prothrombin concentration. On the other hand, the method described in this paper is not affected by factors influencing the conversion rate of prothrombin to thrombin.

An objection against the author's method may be that the introduction of alcohol changes the activity of thrombin in its action upon fibrinogen, and may also affect the yield of thrombin from the prothrombin originally present. Nevertheless, the method gave higher results in one case of dicumarol hypoprothrombinemia than that of Quick. Furthermore the data in Table II speak against a significant diminution of the yield of thrombin due to the introduction of alcohol.

An advantage of Quick's method is the large amount of clinical experience that has been obtained with it. The safety limits for the administration of dicumarol are defined by it, and although this definition may be arbitrary and not correspond to actual prothrombin concentration, the method has been a satisfactory guide in the treatment of thrombo-embolic disease.

No attempt has yet been made to isolate purified thrombin from "thrombinized" plasma in which the antithrombic activity has been suppressed by alcohol. But the crude product obtained by adding whole blood to alcohol, and converting subsequently the prothrombin to thrombin with the aid of human milk thromboplastin as described above, has repeatedly been given by topical application to a hemophiliac patient who is suffering from frequent attacks of serious epistaxis resistant to adrenalin and tamponage. The result—in this only case—was an immediate cessation of the bleeding, which otherwise would have required substitutional blood transfusion.

SUMMARY.

(1) The antithrombic activity of plasma has been suppressed by the use of a 25 per cent concentration of ethyl alcohol. Such alcohol plasma had a remarkably stable thrombic activity after addition of calcium and thromboplastin.

(2) A simple method for the rapid production of crude thrombin has been described.

(3) A convenient two-stage method for the determination of prothrombin has been developed. The procedure involves a suppression of the antithrombic activity of defibrinated oxalate-plasma. Prothrombin is subsequently converted to thrombin by calcium and human milk (thromboplastin). Finally, various dilutions of thrombin containing plasma are tested for thrombic activity with constant amounts of fresh normal human plasma (fibrinogen).

(4) Results obtained by this method have been compared with those obtained by the method of Quick, and the implications of the method outlined have been discussed.

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THE OEDEMATIENS TOXIN ANTITOXIN REACTION: WITH SPECIAL ATTENTION TO THE NATURE OF THE SERA EMPLOYED.

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SINCE Danysz (1902) showed that ricin was precipitated by antiricin serum, *in vitro* antigen-antibody reactions of all types have been extensively studied (see reviews by Marrack, 1938; Boyd, 1943; Kabat, 1943; and Landsteiner, 1945). The toxin antitoxin flocculation reaction first noted for diphtheria by Ramon (1922) was soon extended to other similar reactions. Its usefulness is limited by the toxin strength of the particular culture filtrates made, as very weak culture filtrates in the presence of their antitoxin will not produce sufficient floccules to be seen with the naked eye. The possibility of several antigens being present in the same culture filtrate may also lead to misleading flocculation results for any particular antigen unless a preliminary titration using an antigen of known potency as a blend is used (Anderson, Brown and MacSween, 1945). In recent years the flocculation reaction has been used as a preliminary to animal tests in titrating gas gangrene antitoxins. In these laboratories, for instance, after June, 1942, all oedematiens sera were titrated by flocculation with an oedematiens toxoid, and toxins were titrated against a refined serum (pepsin concentration method—Pope, 1938, 1939). In 1943 it was found necessary to titrate for toxin some unsterile supernatants obtained by centrifuging whole oedematiens cultures in a high speed Bara-Gyro centrifuge. The refined serum normally used as a standard appeared to be quite unsuitable for this work; other similarly refined sera were tried unsuccessfully. No point of optimum flocculation could be determined either by Marrack α or β methods. Several natural sera were, therefore, tried as standards. These were chosen for investigation as having a moderately short time of flocculation with a test toxoid (a formalized culture filtrate), and as being available from horses which were undergoing their first course of immunization. Of three sera chosen only one was found to flocculate sufficiently quickly with unformalized toxic cultures to make a flocculation test practicable. The other two flocculated much more slowly with toxic culture filtrate than with the original test toxoid. It was noted that the serum chosen as a standard serum had a very much higher *in vivo* antitoxic value than *in vitro* (flocculation) value (Glenny, 1931; Barr and Glenny, 1931). An investigation of the differences in the toxin-antitoxin reaction with similar high ratio sera and with normal ratio and low ratio sera was therefore undertaken.

METHODS AND RESULTS.

I. *The Determination of Serum Concentration-flocculation Time Curves for the Flocculation of Various Sera and Fractions of these Sera against a Constant Potency of Toxin and the Corresponding Toxoid.*

A number of horse serum and plasma samples were chosen from the *first* bleedings taken at the end of the first immunization against oedematiens toxin. These sera were all prepared between June, 1942, and February, 1944. The earlier sera were prepared by the use as antigen of culture filtrates made in Wright's medium fermented with *Aerobacter aerogenes* before seeding with *Clostridium oedematiens* Type A (Scott, Turner and Vawter, 1934). The later sera were prepared by the use of culture filtrates obtained by growing a strain of *Cl. oedematiens* Type B on papain digest of horse muscle supplemented with a protein free muscle extract (Macfarlane and Knight, 1941). The *in vitro* (flocculation) and *in vivo* antitoxic values had all been recorded; in each case the *in vitro* values had been obtained by titration against a toxoid prepared on the same type of medium and with the same culture as that used for the immunization. The *in vivo* values had been obtained by the routine method of testing oedematiens sera in mice used in these laboratories as follows: Varying dilutions of the sera were mixed with a test dose of toxin as determined against the laboratory standard serum. The mixtures were made up to such a volume that each mouse received one L + dose of toxin injected subcutaneously in 0·5 ml. of solution which had been standing for 1·5 hr. The dilution of serum which protected 50 per cent of the mice against this dose of toxin was taken as the end point, i.e. it contained one unit of antitoxin. Each sample was tested four or five times, first at wide differences and finally at 10 per cent differences, using two mice for each dilution of serum.

The curves recorded in Fig. 1, 2 and 3 were obtained as follows: A volume of toxin containing 10 Lf doses [as titrated against both the standard natural (unconcentrated) serum and the standard refined (pepsin-process) serum, thus to identify the *specific* flocculation zone], was delivered into each of thirty small tubes. Dilutions of serum made in 1 per cent NaCl containing borate (0·45 per cent) as preservative were added to the toxin so that the serum concentration differed by approximately 20 per cent between each tube. The mixtures were made up to a constant volume (2·0 or 2·5 ml.) with the same diluent. It was arranged that the tube containing the first flocculating mixture of the specific zone was towards the middle of the dilution range. A similar set of tubes was prepared, using a toxoid prepared from these toxins and adjusted to the same pH value as the toxin. In practice the toxin and toxoid were run out and the serum and saline were added to both in parallel, so that additions were finished at almost identical times. The contents of the tubes were then mixed by inversion and the tubes placed in a water bath at 50°. The contents of the tubes were observed at frequent intervals against a reading lamp and the time of flocculation of the mixture in each tube was noted. When it was necessary to time the mixtures for several days the racks of tubes were put into the cold room overnight and this time was considered as negligible. No racks were put into the cold room unless the mixtures had already been incubated for at least two hours. The logarithms of the concentrations of the sera were plotted against the time in minutes for flocculation to appear. Typical serum concentration-flocculation time curves are given on Fig. 1, 2 and 3 for various sera against the same toxin,

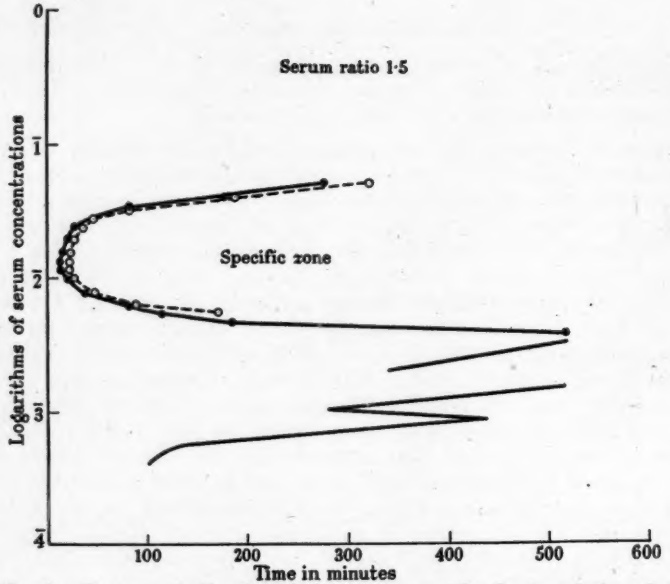

FIG. 1.—Showing the concentration-flocculation time curves for the flocculation of plasma Ex 921 (high ratio) against Toxin 1 and Toxoid 1 (see Table I).
---- Toxoid curve. —— Toxin curve.

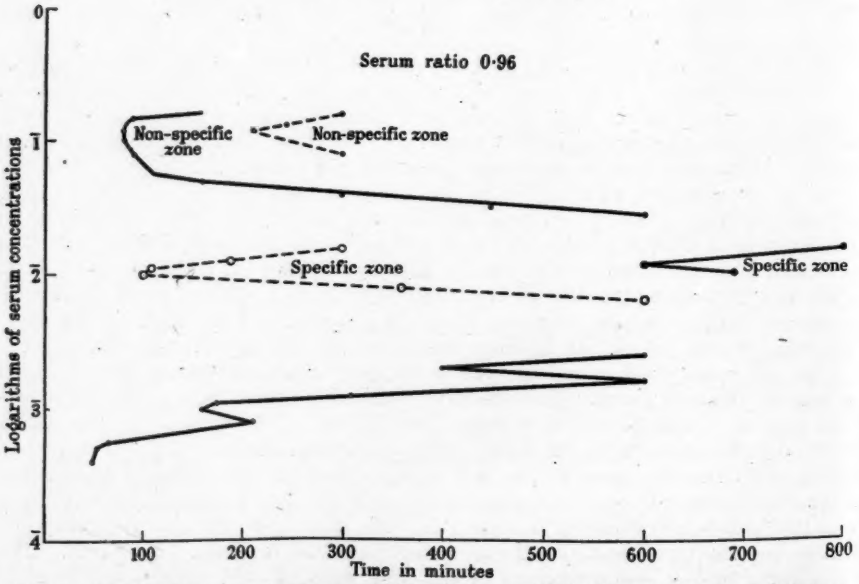

FIG. 2.—Showing the concentration-flocculation time curve for the flocculation of serum NUX 7 (normal ratio) against Toxin 1 and Toxoid 1 (see Table I).
---- Toxoid curve. —— Toxin curve.

and Table I gives the specific flocculating times for a series of sera against two toxins and their corresponding toxoids. It will be noticed that the curves in Fig. 1 for the high ratio serum are quite different from those in Fig. 2 and 3.

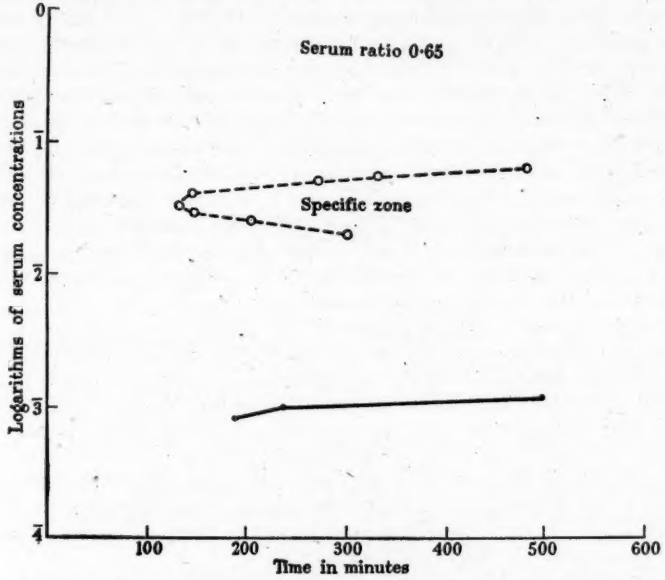

FIG. 3.—Showing the concentration-flocculation time curve for the flocculation of plasma Ex 747 (low ratio) against Toxin 1 and Toxoid 1 (see Table I).
- - - - Toxoid curve. —— Toxin curve.

TABLE I.—*Showing the Times of Flocculation of a Number of Oedematiens Sera Against Two Toxins and Their Corresponding Toxoids.*

Serum or plasma.	Antitoxic value.		Serum ratio.	Time of flocculation in minutes against—			
	In vivo units.	*In vitro* units.		Toxin 1.	Toxoid 1.	Toxin 2.	Toxoid 2.
R 8112	1200	600	2·0	25	20	48	50
Ex 921	1500	1000	1·5	15	25	20	25
Ex 914	1800	1125	1·6	95	80	155	135
Ex 913	2000	875	2·28	35	55	65	65
Ex 706	1550	1250	1·24	40	40	65	55
Ex 693	1850	1150	1·61	35	55	45	55
NUX 7	1200	1250	0·96	500	100	880	340
NUX 8	2200	2400	0·92	300	85	735	190
Ex 893	1200	1250	0·96	—	—	650	225
Ex 851	1700	1900	0·89	595	85	325	225
Ex 702	1500	1600	0·94	—	—	365	260
Ex 747	380	580	0·65	∞	135	385	50
Ex 740	900	1450	0·62	∞	120	330	130

The times of flocculation shown in the different figures are not comparable, since although for each serum the corresponding toxin and toxoid curve were timed exactly together, the different sera were done on different occasions and the convection currents were not regulated; the times obtained on one day could, therefore, not be exactly repeated on another. In Fig. 1 the toxin and toxoid curves are practically superimposed and both are broad in shape. All serum dilutions between 1/120 and 1/30 had flocculated 30 min. after the first flocculation began. The exact shape of the curve for a high ratio serum varies with the serum, and the times of flocculation are dependent on the toxin as well as the serum used (Table I). With normal or low ratio sera (Fig. 2 and 3) the toxoid curve is much narrower and there is no specific toxin flocculation for serum Ex 747. When the toxin curve is present as with serum NUX 7, it is clear that the time of flocculation and the range over which the zone extends are very different from those of the toxoid zone. The unusual feature as compared with such toxins as diphtheria and tetanus is that the time of flocculation with oedematiens toxin is so much longer than with the corresponding toxoid.

It is interesting that with the serum NUX 7 there is a wide curve, showing the presence of a fast flocculating antigen-antibody combination containing no antitoxin at approximately twelve equivalents of antitoxin (see Fig. 2, non-specific zone, and Table V). Its counterpart is present in the toxoid curve with an optimal flocculation time slower than that of the specific toxoid-antitoxin combination. Several toxic filtrates gave more points of flocculation with the serum used for Fig. 2 than that shown, and although the experimental serum used for Fig. 3 had a specific zone for toxoid and no toxin flocculation, with other toxic filtrates both specific and non-specific zones were obatined. High ratio sera rarely gave multiple flocculation zones.

Flocculation with serum fractions.

A series of serum fractions was prepared from one normal ratio serum and one plasma by the method of Barr, Glenny and Pope (1931). A fraction obtained with larger amounts of ammonium sulphate was also prepared from a high ratio plasma. Serum concentration-flocculation time curves were plotted for all these

TABLE II.—*Showing the Times of Flocculation of Ammonium Sulphate Fractions with Toxin 2.*

Serum.	Precipitation limits ammonium sulphate. G. per litre.	Antitoxic value. *In vivo* units.	Antitoxic value. *In vitro* units.	Serum ratio.	Time of flocculation in minutes against— Toxin 2.	Time of flocculation in minutes against— Toxoid 2.
NUX 7	0–160	Insufficient precipitate to test.				
(serum)	160–190	300	250	1·20	100	200
	190–220	250	260	0·96	220	440
	220–300	400	500	0·80	1900	960
Ex 851	0–160	340	370	0·97	∞	440
(plasma)	160–190	145	140	1·03	220	200
	190–220	320	375	0·85	580	400
	220–300	150	180	0·83	∞	5000
Ex 921	220–300	150	115	1 30	1140	640

fractions to determine whether the property of fast flocculation with filtrates was due to antitoxic protein precipitated by the lower concentration of ammonium sulphate which would contain the γ globulin antitoxic fraction according to Kekwick, Knight, Macfarlane and Record (1941). Table II shows the antitoxic values, the serum ratios, and the times of flocculation against a particular toxin and its corresponding toxoid for all these fractions. It is clear that the property of fast flocculation is linked with a protein precipitated by the lower concentrations of ammonium sulphate, but not the lowest. This is in all probability the γ antitoxic globulin of Kekwick *et al.* (1941). It is also clear that for the oedematiens antisera it is not this same protein which wholly accounts for high *in vivo*/*in vitro* ratios.

II. *The Determination of the Nitrogen Precipitated by Different Types of Sera at Varying Dilutions from a Constant Potency of Toxin and its Corresponding Toxoid, also of the* in vivo *Units of Antitoxin Remaining in the Supernatant after Precipitation with Toxin.*

It was noticed while determining the serum concentration-flocculation time curves that more protein appeared to be precipitated at the *in vitro* optimal point from high ratio sera than from normal sera. The following nitrogen determinations were therefore made:

Two series of dilutions, one from a serum having a high *in vivo*/*in vitro* ratio and one from a serum having a normal ratio were mixed with 100 Lf doses of two different toxins and their corresponding toxoids. In every case the mixture was made up to a volume of 13 ml., and was incubated at 50° for two days.

TABLE III.—*Showing the Nitrogen Precipitated by Varying Amounts of Serum NUX 7 with 100 Lf Doses of Toxins AE 790 and AE 889 and Their Corresponding Toxoids ME 790 and ME889.*

In vivo antitoxic units.	Average amounts of nitrogen precipitated in mg. from—				*In vivo* units of antitoxin combined with 100 Lf of toxins—	
	ME 790.	AE 790.	ME 889.	AE 889.	AE 790.	AE 889.
48	None	None	0·0374	0·0483	..	..
60	0·0735	0·0354	0·0884	0·0721	..	..
86	0·1233	0·0901	0·1742	0·0929	..	..
108	0·1332	0·1178	0·1841	0·1314	..	..
132	0·1624	0·1294	0·1968	0·1249	..	..
168	0·2181	0·1584	0·1792	0·1320	>118	..
192	—	—	0·1774	0·1505	..	..
216	0·2084	0·1149	—	—	>166	..
240	—	—	0·1333	0·1752	..	>190
300	None	..	0·0626	0·1300	..	222
360	..	..	—	0·0176	..	243
420	..	..	—	None	..	264

———— Equivalent quantities of toxin and antitoxin.
..... Optimal flocculation point.
In vivo antitoxic value of serum, 1200.
In vitro antitoxic value of serum, 1250.
Serum ratio, 0·96.

The floccules were spun out of the mixtures, washed twice with saline, dissolved by adding N-NaOH and their nitrogen content estimated by the micro-Kjeldahl method. Each dilution of serum was set up in duplicate, and when inconsistent results were obtained were repeated in duplicate or triplicate. The results were satisfactorily consistent except in that part of the range where re-solution of the precipitate began. The abnormalities were more apparent with toxoids than with toxins, and an average value of the results obtained is given, although it is realized that this value may have no great numerical significance. The toxin itself gave a precipitate when centrifuged, and the nitrogen corresponding to this was subtracted from the total floccule nitrogen. No such blank was found with toxoids. The supernatants from the toxin floccules were tested to determine the amount of antitoxin (measured by the *in vivo* test), which was combined with 100 Lf doses of toxin. These results are shown in Tables III and IV.

TABLE IV.—*Showing the Nitrogen Precipitated by Varying Amounts of Serum R* 8112 *with* 100 *Lf Doses of Toxins AE* 790 *and AE* 889 *and Their Corresponding Toxoids ME* 790 *and ME* 889.

In vivo antitoxin units.	Average amounts of nitrogen precipitated in mg. from—				*In vivo* units of antitoxin combined with 100 Lf of toxins—	
	ME 790.	AE 790.	ME 889.	AE 889.	AE 790.	AE 889.
72	None	0·1042	0·1235	0·1166	..	..
96	0·1371	0·1263	—	—	..	..
108	0·1484	—	0·2312	0·1637	..	..
120	0·1701	0·1442	—	—	..	..
144	0·1692	0·1501	0·2766	0·1888	..	..
192	—	—	0·3207	0·2382	..	>142
216	0·2335	0·2636	—	—	—	..
360	0·3679	0·4649	0·4691	0·4406	301	301·5
420	0·4052	0·4826	—	—	345	—
432	—	—	0·5089	0·5112	—	348
480	0·4866	0·5921	—	—	383	—
660	—	—	0·5711	0·6667	—	426
860	0·4827	0·7558	0·5159	0·6692	526	457
1080	—	—	0·4336	0·6812	—	482
1200	—	0·7534	—	—	526	—
1560	0·1436	0·5239	0·0842	0·4067	520	429
1800	—	..	—	0·2913	—	435

———— Equivalent quantities of toxin and antitoxin.
..... Optimal flocculation point.
In vivo antitoxic value of serum, 1200.
In vitro antitoxic value of serum, 600.
Serum ratio, 2·0.

It is clear that for each serum one equivalent of toxoid precipitates more nitrogen than does one equivalent of toxin. With the normal ratio serum this is the optimal flocculation point, but the difference in the amounts of precipitate formed by the toxin and toxoid are not great enough to account for the much longer time of flocculation of the toxin combination. The nitrogen precipitated

at the optimal flocculation point of the high ratio serum R 8112 is at least twice as much as that from the normal serum, and may account in part for the speed of flocculation of this serum. Further, the maximum amount of nitrogen is precipitated with toxin or toxoid by two and a half equivalents or less of normal ratio serum as compared with six to eight equivalents of high ratio serum. The latter maxima have values two to four times as great as the former, i.e. far more nitrogen is precipitated from the high ratio serum. Mixtures containing three to four equivalents of serum NUX 7 per equivalent of toxin did not flocculate; judging by *in vivo* tests on the supernatants (Table III) 2·5 equivalents of antitoxin were combined. Mixtures containing fifteen to eighteen equivalents of serum R 8112 per equivalent of toxin flocculated to some extent, though *in vivo* tests (Table IV) showed that only four or five equivalents of antitoxin were combined.

III. *The Determination of the Amount of Antitoxin which can be Recovered from the Floccules and from the Floccule Suspension of Mixtures Containing Varying Ratios of Antitoxin to Toxin. Also the Determination of the Antitoxin Content of the Supernatants from these Floccules.*

These experiments were designed to ascertain the solubility of floccules (both from neutral mixtures and from optimal flocculation mixtures), and to obtain confirmatory evidence of the number of antitoxin equivalents combined and precipitated with one toxin equivalent. The normal ratio serum NUX 7 and the high ratio serum R 8112 were used. Floccules were prepared using 1000 Lf doses of toxin AE 924. The ratios of *in vivo* units of antitoxin to toxin are shown with the results in Table V, and are such that for both sera the *in vivo* neutral mixtures and *in vitro* optimal flocculation mixtures are covered. All the mixtures were incubated for two days at 50°. For digestion the floccules were centrifuged, washed twice with saline and digested as recommended by Pope and Healey (1939) modified by Moloney and Hennessy (1942). The digestion was carried out at 37° for 1·5 hr. instead of at room temperature, a much more satisfactory yield being obtained by this modification. For the digestion of a suspension of floccules or of a supernatant from floccules the whole was treated with pepsin as above, precipitated with ammonium sulphate and the precipitate re-dissolved to obtain a solution of suitable potency for testing purposes. All floccule digests were tested for *in vivo* potency as described earlier (see Section I), as were also relevant suspensions and supernatant digests. Supernatants from the larger excesses of serum NUX 7 were titrated for antitoxin, and it may be assumed from the results on the supernatants shown in Table III that no antitoxin would be detected by *in vivo* methods in the others. The tests of supernatants of serum R 8112 in Table V agree with those found for the other toxins in Table IV. The additional *in vitro* results were obtained by independent titrations with a test toxoid, and by blending with a serum of known potency followed by flocculation with a test toxoid.

The results (Table V) indicate that recoveries of antitoxin from suspensions, floccules, and supernatants from neutralized and over-neutralized toxin are of the same order, viz. about 30 to 35 per cent. Under neutralized antitoxin appears to be more difficult to recover. Greater recoveries have been obtained with other oedematiens toxic filtrates. It is clear that the combined antitoxin is completely precipitated from neutral mixtures, from the 1 : 1·8 mixture with

TABLE V.—*Showing the Antitoxin Recovered by Peptic Digestion from Floccule Suspensions in Toxin and in Antitoxin, from the Floccules Themselves, and the Antitoxin Remaining in the Supernatants.*

	Ratio.	Total units of antitoxin.									
	Toxin antitoxin.	In original mixture. *In vivo* units.	Recovered from floccule suspension. *In vivo.*		Recovered from floccules. *In vivo.*		*In vitro* units.	Free antitoxin in supernatants. *In vivo.*		*In vitro* units.
			units.	%	Units.	%		Units.	%	
Serum NUX 7; Serum ratio 0·96	1 : 0·36	360	170	23·5	No floccules		No floccules	Not tested		Not tested
	1 : 1·00*†	1,000	340	34·0	330	33·0	Not tested	„	„	„
	1 : 1·80	1,800	520	29·0	570	31·5	„ „	„	„	„
	1 : 3·60	3,600	—	—	170	4·5	„ „	17,50‡	48·5	„
	1 : 12·00	12,000	—	—	<200	<2·0	Peculiar floccules	12,000	100·0	14,000
Serum R 8112; Serum ratio 2·00	1 : 1·0*	1,000	—	—	345	34·5	140	<400	—	None
	1 : 2·0†	2,000	—	—	720	36·0	334	<400	—	„
	1 : 10·0	10,000	—	—	1,650	16·5	990	4,550	45·5	1,770

* *In vivo* neutral point.

† *In vitro* optimal flocculation AE 924 toxic filtrate employed.

‡ 29 per cent of the total antitoxin was recovered by peptic digestion from this supernatant, making an overall recovery of 33·5 per cent from this toxin-antitoxin mixture.

serum NUX 7 and from the 1 : 2 mixture with serum R 8112. With the 1 : 10 mixture and serum R 8112 about half the antitoxin must be precipitated and only half combined (see results on supernatants). Almost all the combined antitoxin (i.e. 5 equivalents) must be precipitated.

DISCUSSION.

The fast flocculating properties of high ratio sera have been known since the early days of toxin-antitoxin flocculation and have been discussed by Glenny (1931). On the other hand, the faster flocculation of normal ratio oedematiens sera with toxoid than with toxin appears to be a unique property of this antitoxin. It is interesting that when normal ratio sera were fractionated, the fast flocculating fractions were not necessarily high ratio fractions. Also the high ratio fraction from a high ratio serum was not a typically fast flocculating fraction. Evidently, as suggested by Kekwick *et al.*, there is a larger proportion of fast flocculating antitoxin in most high ratio sera than in normal and low ratio sera. High ratio sera alone are suitable as standard flocculating sera for titrating toxoids and toxins.

The comparative amounts of floccules precipitated from high ratio and normal ratio sera shown by the nitrogen results and the combination of the antitoxin as shown by the *in vivo* tests on the supernatants show marked differences (Table III and IV). At $3\frac{1}{2}$ equivalents of antitoxin the normal ratio serum gave very little precipitate and only $2\frac{1}{2}$ equivalents of antitoxin were combined, while the high ratio serum gave a large precipitate and about 3 equivalents of antitoxin were combined. Mixtures containing more than 4 equivalents of normal ratio sera did not flocculate, although combination of $2\frac{1}{2}$ equivalents of antitoxin was shown, while with up to 12 equivalents of high ratio serum increasing amounts of nitrogen were precipitated, and a maximum of 5 equivalents of antitoxin was combined. At 12 equivalents of normal ratio serum a second zone of precipitation occurred, but no antitoxin was precipitated or combined (Table V). The yield of antitoxin by the peptic digestion used was about 30 per cent when antitoxin was in excess, whether the antitoxin was flocculated, combined or free (Table V and in the text of Section 3). If the results of the digestion of the floccules from the high ratio serum are viewed in the light of this fact it is clear that almost insoluble floccules containing 1, 2 and 5 equivalents of antitoxin were prepared. With 10 equivalents of serum R 8112 to 1 equivalent of toxic filtrate AE 924 a practically insoluble toxin antitoxin compound (one part toxin to five parts of antitoxin) appeared to be in equilibrium with an excess of 5 equivalents of antitoxin. Addition of between 8 and 12 equivalents of antitoxin caused maximum nitrogen to be precipitated by both toxins and approximately 5 equivalents of antitoxin are combined, the solubility of the 5 equivalent compound formed depending to some extent on the toxin from which it is prepared (Table IV). Ultracentrifuge investigation of the diphtheria toxin-antitoxin combinations led Pappenheimer, Lundgren and Williams (1940) to believe that not more than eight molecules of antitoxin could combine with one molecule of toxin. They also deduced from other data that two molecules of antitoxin were equivalent to one molecule of toxin. The compound containing four equivalents of antitoxin to one equivalent of toxin was thus the maximum combination which they foretold. It is interesting that both from the supernatants of floccules and from floccule digestion, experimental evidence has now been

obtained that five equivalents of oedematiens antitoxin can combine with one equivalent of toxin, i.e. a compound TA_{10} can be formed. The combining power of other antigens and antibodies which have been studied in antibody excess are hardly comparable as the antibodies have been prepared in rabbits.

It is difficult to suggest a reason for the higher nitrogen content of toxoid than toxin floccules at the equivalent point, particularly as this occurs with both types of sera. It might be suggested that the toxin floccules were more soluble than the toxoid floccules, and that this also accounts for the longer time of flocculation with normal ratio sera. If this were assumed, then the equal rates of flocculation of a high ratio serum with toxin or toxoid could still be accounted for by the presence of an unusual amount of γ globulin antitoxin. On the other hand, it may be that the amount of precipitable protein is increased when formaldehyde is added to the toxin. Both of these suggestions, however, apply solely to the equivalence point; at other concentrations of antibody the toxin floccules do not necessarily contain less nitrogen. It is also difficult to understand the shift of the optimal flocculation point, although maximum combinations of 5 and $2\frac{1}{2}$ equivalents of antitoxin corresponding to optimal flocculation equivalents of 2 and 1 are suggestive. Evidently the antibody of the high ratio serum forms solid combinations more readily than that of the normal ratio serum. The suggestion put forward by Barr and Glenny (1931) for diphtheria antitoxin "that in high ratio sera only a fraction of the total antitoxin flocculates with toxin *in vitro*" appears to need further consideration. From the evidence already discussed it is clear that practically complete precipitation of both normal and high ratio sera occurs to an excess of one equivalent of antitoxin. At 12 equivalents of normal ratio antitoxin the supernatant serum ratio is slightly decreased, probably not significantly, while at 10 equivalents of high ratio antitoxin the supernatant serum ratio is definitely increased and that of the floccules is decreased (Table V). It is clear, therefore, that a proportion of non-flocculating antitoxin is present in a high ratio serum. It is also clear that far more antitoxin is precipitated by toxin from a high ratio than from a normal ratio serum, in the presence of a large excess of antitoxin.

SUMMARY.

1. It has been shown that normal ratio oedematiens sera flocculate much more readily with toxoid than with toxin. High ratio sera flocculate equally readily with both and are, therefore, much more suitable for standard sera for the *in vitro* estimations of toxins and toxoids.

2. The fast flocculating antitoxin appears to have the salting-out properties of γ antitoxin, and is not necessarily associated with a high *in vivo*/*in vitro* ratio.

3. Evidence has been given for the existence of a compound TA_{10} formed from a high ratio oedematiens antitoxin combined with toxin.

4. It has been shown that from mixtures containing a large excess of antitoxin, high ratio oedematiens antitoxin has greater ability to combine and to flocculate than normal ratio antitoxin.

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THE NATURE AND MODE OF ACTION OF THE INTRAVENOUS TOXIC FACTORS IN SALINE EXTRACTS OF MUSCLE AND OTHER TISSUES.

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FROM the dawn of experimental medicine the fact that tissue extracts, when injected intravenously, produce harmful and often fatal effects has been recognized. Obviously this has led to unending confusion between non-specific and specific effects, and to many wrong conclusions in every branch of experimental medicine. The advent of biochemistry with the resulting isolation of biological substances was no doubt hastened by the anomalous and often seemingly contradictory results of injecting extracts of every kind of tissue. Investigations described here will, it is hoped, help to resolve some of these difficulties, and show in part how they can be overcome.

This work is a direct consequence of earlier observations on adenosine triphosphate shock (Green, 1943; Bielschowsky and Green, 1943). The muscle

extract originally used in that work was prepared according to the method of Dyckerhoff, Schörcher and Torres (1939), and is essentially an alcohol-precipitated product from acteone dried muscle. It was from this product that adenosine triphosphate was isolated, and shown to be responsible for the lethal effect on intravenous injection in the rabbit described by Dyckerhoff. It was, however, found that adenosine triphosphate when injected in adequate amounts by routes other than the intravenous, produced not rapid death, but the gradual onset of a shock-like state resembling that seen after prolonged limb ischaemia. It was this effect rather than the intravenous which was of great interest, because of its possible connection with the mechanism of shock. There have been many developments in this work (Green and Stoner, 1944; Stoner and Green, 1944; Green, 1945; Stoner and Green, 1945*a*; Stoner and Green, 1945*b*; Bielschowsky and Green, 1945; Bielschowsky, Green and Stoner, 1946), but since a mass of observations (a few of which are referred to in the present paper) is now being assembled for a full report, this aspect will not be considered in detail here. In the present work we are concerned with the role of adenosine triphosphate and related compounds in the toxic effects of tissue extracts when injected intravenously.

Lenggenhager (1935) ascribed the lethal effects of freshly prepared serum (*fruhgift*) and saline extracts of chopped muscle, when injected intravenously, to the presence of thrombin, and found thrombi in the lungs and right heart following death from these extracts. Dyckerhoff and Schörcher (1939) investigated the nature of the clotting factor in muscle extract and found no thrombin, but considerable thrombokinase activity. If an anticoagulant was given before the injection of fresh muscle extract, up to five times the lethal dose was tolerated. They also found that, whilst the thromboplastin content of the extract did not fall, the intravenous toxicity fell rapidly on standing and at 2½ hours had disappeared. If, however, the inactive muscle extract was injected together with some of the product (myotoxin) prepared from acetone dried muscle the lethal potency was restored, and the rabbit died with extensive clots in the pulmonary arteries and right heart. It was therefore concluded that the lethal effect of a fresh muscle extract was due to a combination of thrombokinase and an unknown lethal factor in muscle which, by itself in much higher doses, was lethal on intravenous injection. This work was done exclusively in the rabbit, and in broad outline we confirmed it at an early stage in our shock investigations. When it was realized that clotting factors coming from injured tissue might well play a part in the complex etiology of shock, a detailed investigation into the possible relationship between the biological effects of adenosine triphosphate and various blood-clotting factors was undertaken. Some of the preliminary results are described here.

METHODS.

Experiments were performed on over 300 rats under light ether anaesthesia. The test substances were injected at the rate of 1 ml. in 10 seconds into the femoral vein, the respiratory rate and excursions being recorded. In 90 rats direct observations on the cardiac rate were made by visual observation of a fine straight needle inserted into the left ventricular wall through the intercostal space. In 36 rats the blood pressure was determined by the direct method as described by Griffith and Jeffers (1942), except that, since the effect of clotting factors was being investigated, the animal could not be given heparin.

In 23 cats, anaesthetized with nembutal, the carotid blood pressure was recorded by a mercury manometer and the respiratory movements by a tambour attached to a partly inflated sphygmomanometer cuff wrapped round the thorax. Test substances were injected into the external jugular vein.

The fresh muscle extract (F.M.E.) was a filtrate prepared under ice-cool conditions from finely scissor-minced rat muscle ground up with two volumes of 0·85 per cent saline. The other tissue extracts were prepared similarly, and are referred to as fresh when injected within 15 min. of removing the tissue. These extracts, after standing for 3 hours at room temperature, are referred to as old extracts (e.g. O.M.E.—old muscle extract).

Other sources of thromboplastin used were a saline extract of acetone dried rabbit brain (Quick, 1942) and Russell Viper Venom. One unit of thromboplastin is the amount required to clot, in 60 seconds at 37° C., 0·1 ml. of oxalate rabbit plasma recalcified by the addition of 0·1 ml. 0·02M $CaCl_2$; total volume of reaction mixture, 0·3 ml. Thrombin was given in a relatively pure form prepared from bovine serum (Parke, Davis).

Heparin, the main anticoagulant used, was injected in amounts of 1 mg./100 g. rat. In some experiments 5 mg./100 g. of the anti-prothrombin substance 3 : 3′-methylene-bis-4-hydroxycoumarine was given by mouth 24 hours prior to the test.

Adenosine triphosphate (ATP) and inosine triphosphate were given as the Mg. salts.

The term lethal dose is used arbitrarily, being the minimal amount lethal to all of a group of 4 rats, i.e. roughly approximate to the $L.D._{100}$. Where it varied widely in different groups the range is given. All doses are expressed in terms of a 100 g. rat.

Fresh Muscle Extract (F.M.E.).

Fresh muscle extract (0·2–0·5 ml. F.M.E.) produces profound effects in the rat. Respirations usually increase greatly in depth and rate and, after a period of from 5 to 15 seconds, cease entirely in expiration. This period of apnoea varies with the dose, and has been observed to last 160 seconds with ultimate recovery. Commonly if the period is prolonged more than 90 seconds respiration never fully recovers and a variable period of asphyxial gasping precedes death. If the rat ultimately recovers the resumed respirations are slow and shallow, returning to normal over a period of 2–4 minutes. When a fatal issue is only delayed the respirations continue slow, forceful and irregular with occasional periods of apnoea until occasional gasps indicate the approach of death, which, though usually occurring in from 10–60 minutes, may be delayed for several hours. Throughout this period the rat, though conscious, is semi-prostrate.

The heart momentarily stops after the injection of F.M.E., and then beats very slowly. With non-lethal doses (0·1–0·4 ml.) the rate returns to normal in 1–4 minutes, but with a rapidly lethal dose (0·5 ml.) the recovery of the heart is never complete, though the rate always increases considerably from the initial low level and may attain half or more of the normal, even when respirations have entirely ceased. One curious feature of the heart rhythm often observed after lethal doses is a rapid alternation in rate, rapid acceleration occurring for short periods. After lethal doses the heart continues beating with slowly declining force and rate for periods up to 10 minutes. When the survival time is prolonged the heart often appears to recover completely and the rate remains

in the normal range until respiration finally ceases. The initial bradycardia is associated with a fall in blood pressure (Table I).

TABLE I.—*Effect of F.M.E. on the Blood Pressure of the Rat.*

Age of extract (min.).	Effect of 0·2 ml. F.M.E. per 100 g. body weight; % fall in B.P.	Equivalent dose of MgATP mg./100 g. body weight.
15	50	0·2
30	43	—
60	25	—
180	29	0·025

After lethal doses gross evidence of the cause of death was not found. There was often, but not always, some loose clot in the right ventricle but no gross clotting in the pulmonary arteries. The lung expansion varied from full expiration to inspiration, and patchy areas of congestion and a few superficial haemorrhages 1–3 mm. in diameter were seen and were more widespread when death was delayed. Otherwise the findings were merely those of acute congestion of the liver and kidneys associated with right heart dilatation, changes which were regarded as terminal and non-specific. The histological findings were similar to those described later following lethal doses of ATP and thrombin.

TABLE II.—*Intravenous Lethal Doses/100 g. Rat of Various Saline Tissue Extracts and other Coagulants With and Without Added Magnesium Adenosine Triphosphate.*

Coagulant.	Alone.		Combined (1 mg. ATP).	
	Lethal dose ml./100 g. body weight.	Thrombo-plastin units.	Lethal dose ml./100 g. body weight.	Thrombo-plastin units.
F.M.E.	0·5	90	0·1	18
Heated 60° C. × 15′	>1·5	>5	1–1·5	3–5
O.M.E.	0·5–1·5	90–270	0·1	18
Heated 60° C. × 15′	>1·5	>5	1–1·5	3–5
F.L.E. (lung)	0·15	140	0·03	28
Heated 60° C. × 15′	>1·0	—	0·5–1·0	—
O.L.E.	0·15–0·3	140–280	0·03	28
F.T.E. (testis)	0·3	150	0·05	25
O.T.E.	0·3–0·8	150–400	0·05	25
F.B.E. (brain)	0·5	125	0·1	25
O.B.E.	0·5–1·0	125–250	0·1	25
F.K.E. (kidney)	0·45	150	0·1	30
F. liver E.	>1·0	>50	0·5	25
Brain thromboplastin	0·5–1·5	150–400	0·1	30
Russell Viper Venom (1/10,000)	1·0–3·0	175–525	0·2	35
Thrombin	50–100 Iowa units	—	7·5	—
Trypsin	—	—	>1 mg.	—

F.M.E. can be rendered almost completely innocuous by the previous intravenous injection of heparin. At least 3 and up to 6 times the lethal dose can then be tolerated. The only obvious signs are temporary quickening of the respiratory rate, sometimes preceded by apnoea of a few seconds' duration. There is, however, a fall in blood pressure and bradycardia of approximately the same degree and duration as in the unheparinized rat.

The toxicity of F.M.E. can be largely removed by heating for 15 minutes at 60° C., the filtrate being non-lethal in high dosage (Table II). After such treatment its only obvious effect is to increase temporarily the rate and amplitude of respiration and to produce a mild bradycardia.

Old Muscle Extract (O.M.E.).

F.M.E. gradually loses some of its potency on standing at room temperature, and after 1½–3 hours it may be relatively harmless to the rat at the previously lethal dosage. The lethal dose, instead of being fairly constant around 0·5 ml., now varies greatly (see Table II). Doses ranging up to 2 ml. may be tolerated without apparent discomfort except for temporary respiratory stimulation. Lethal doses, as with all clotting agents observed, produce respiratory paralysis following initial stimulation, and if respiration is more than temporarily resumed the rat often remains in a prostrate state with slow and laboured breathing for up to an hour or more. Following lethal doses the autopsy findings differed in no way from those seen after F.M.E.

The complete biological action of the original F.M.E. can be restored by adding sub-lethal amounts of ATP to O.M.E. immediately prior to injection. The minimal amount required for 0·5 ml. O.M.E. is 0·25 mg. ATP (lethal dose, 4 mg.). Smaller volumes, well below the lethal range, become lethal when more ATP is added (e.g. 0·1 ml. O.M.E. + 1 mg. ATP). There is, within limits, a reciprocal relationship between the two agents, but a minimal amount (0·05 ml.) of O.M.E. is required, and this proves fatal with 2 mg. ATP. Equivalent doses on a weight for weight basis are also fatal to the cat. Since the biological effects of the combined agents are very similar to those following ATP + thrombin they are considered in more detail in the next section.

Other Clotting Agents and ATP.

The fact that heparin antagonizes the harmful effects of muscle extract points to the dominant factor being a clotting agent and this proved to be thromboplastin ; thrombin is absent (Stoner and Green, 1947). If other sources of thromboplastin when mixed with ATP produce similar effects, then the hypothesis that the lethal effect of F.M.E. is due to the combined action of ATP and thromboplastin becomes a reasonable one. It was in fact found that both a brain thromboplastin extract and Russell Viper Venom approach the activity of O.M.E. in this respect when equivalent concentrations of thromboplastin are compared (Table II).

That the action is not peculiar to extracts rich in thromboplastin was shown by using another clotting agent—thrombin. Thrombin mixed with ATP reproduces the complete syndrome following F.M.E. Because of its purity and uniform potency it was mainly used in later experiments designed to find the mechanism of this combined action. In this connection it was interesting to

find that the feeding of 3 : 3′-methylene-bis-(4-hydroxycoumarin) in amounts sufficient to cause a three-fold increase in the Quick prothrombin time significantly reduces the toxicity of O.M.E. + ATP but does not affect that of thrombin + ATP, a difference no doubt due to the fact that the principal clotting agent in muscle is thromboplastin.

It will be noted (Table II) that the lethal dose of thrombin combined with ATP is at most 1/7 of the uncombined. As with O.M.E., the limiting factor in the combination is the clotting agent. Below about 2 units of thrombin no marked accentuation of the ATP effect is seen, whereas in the presence of adequate thrombin the activity of ATP in very low dosage is greatly increased. For instance, with 10 units of thrombin 0·2 mg. ATP may on occasion be lethal (4 mg. uncombined ATP), and a very definite effect may be noted with as little as 0·025 mg.

It will be obvious that these results represent very much more than a summation of effect, and an analysis of the biological responses in the cat and rat suggests that the effect of one or other agent on the respiratory system is much enhanced. As with F.M.E., death following ATP + thrombin appears to be due primarily to respiratory failure. In the cat respirations stop immediately and the blood pressure falls precipitously and does not recover. In the rat and cat thrombin is itself a depressor substance as it is in the dog and rabbit (Tagnon, 1945), but in sub-lethal combination with ATP it does not appreciably alter the immediate depressor effect of ATP, though it may prolong the recovery phase. Under these conditions, further doses of ATP in amounts usually easily recovered from are associated with a progressive decline in blood pressure. A similar sequence is seen in the rat, and following a dose of thrombin (e.g. 10 units), which by itself produces a large but transient fall in blood pressure and some respiratory quickening, the sensitivity to ATP is much increased for at least 30 minutes. There is little doubt that thrombin in sufficient amounts accentuates the cardiovascular effects of ATP, but the effect is not nearly so profound as it is on the respiratory system. In the cat, amounts of thrombin which have no influence on the cardiovascular effects of ATP prolong the period of respiratory inhibition considerably. In the rat the effect is still more striking and has been repeatedly observed. The immediate period of apnoea (e.g. 5–10 seconds with 0·5 mg. ATP) is increased greatly when thrombin is combined (e.g. 30–120 seconds with 5 units of thrombin). Moreover respiratory distress, just as after F.M.E., often continues for long periods with the rat in a semi-prostrate state. This shock-like state, which continues for long after the heart rate has returned to normal, is a striking feature when compared with the very transient effect of either agent given alone.

After lethal combined doses the autopsy findings differ in no way from those described following lethal doses of F.M.E. Histological examination of the lung, brain, kidney and liver shows no definite evidence of thrombosis in any vessels but those of the lung, where fibrin thrombi are present in large numbers in the capillaries and smaller arteries and veins. The number of vessels affected is very much greater than with a similar dose of thrombin alone (which is non-fatal), but it is obvious that the circulation in the lung is not completely occluded. Moreover, a very similar picture is seen for some hours after the injection of sub-lethal doses of the combined agents, though by 24 hours many thrombi are retracting and fibrinolysis is occurring. It seems unlikely therefore that thrombosis in the pulmonary vessels is the primary cause of death.

Previous heparin treatment prevents the serious effects of ATP + thrombin. The circulatory depressant effect of thrombin, unlike that of O.M.E., is removed, but the transient effect of ATP on the respiration and circulation is unaltered.

ATP in Fresh Muscle Extract.

Although combined ATP and clotting agent reproduces the effects of F.M.E., it cannot be assumed that ATP is the only other active factor in this extract. That it is one factor is almost certain, as muscle is a rich source of ATP, and if ATP breakdown is prevented by the rapid immersion of the muscle in acetone, the saline extract, My_a (Bielschowsky and Green, 1943) of the dried muscle restores the potency of O.M.E. to that of F.M.E. The effective amount of My_a is almost exactly equivalent to its ATP content (which is around 0·5 per cent). Though there is at least ten times the amount of ATP in muscle required to account for the activity of F.M.E., the practical point is whether sufficient survives mincing and extraction prior to injection. Only an indirect estimate of the ATP content of the extract can be made. Biological assays of the adenosine equivalent and chemical determinations of the acid labile P together indicate a concentration of from 0·07–0·09 per cent ATP in rat F.M.E. prepared very rapidly under ice-cool conditions (Table III). Its depressor activity assayed

TABLE III.—*Adenosine Equivalent and Phosphorus Distribution in Fresh Tissue Extracts. Results in mg./100 ml. Saline Extract* (1 : 2).

Tissue.	Total acid-soluble P.	Inorganic P.	7 min. acid-labile P.	Adenosine equivalent.*	Maximum amount MgATP possible.
Muscle (F.M.E.)	45·7	22·0	10·7	70	93
Brain	23·2	12·5	1·9	45	17
Liver	24·3	7·4	2·1	50	18
Lung	12·4	4·7	0·8	45	7
Kidney	16·7	7·2	0·5	35	4
Testis	16·1	6·1	1·2	30	10

NOTE.—All extracts (except muscle) made from tissues frozen in liquid air immediately on removal.

* See Stoner, H. B., and Green, H. N. (1944).

against MgATP is roughly in line with this estimate (Table I). Roughly two-thirds of the ATP originally present in the muscle has already broken down in a very rapidly prepared extract. Nevertheless, in a lethal dose (0·5 ml.) of F.M.E. the average amount of ATP is at least sufficient to account, together with thromboplastin, for its lethal activity. The initial breakdown of ATP is, however, so rapid that relatively wide variations in samples of F.M.E. over the arbitrary period of 15 min. (see "Methods") are found. The minimal amount of ATP required to render 0·5 ml. O.M.E. uniformly lethal is 0·25 mg., and even in a 15 min. old extract the ATP content of F.M.E. does not fall significantly below this concentration (see Table IV). Breakdown products of ATP must also play some part, for in the rat inosine triphosphate and muscle adenylic acid, combined with thrombin, act similarly to ATP, though in progressively less degree. That other nucleotides may be active in increasing measure in the older extracts is indicated by the fact that the lethal potency does not diminish in direct proportion to the rate of disappearance of ATP (Table IV). After

3 hours at room temperature or at 60° C. for 15 min. probably no active nucleotide remains (Table IV), and the great variability in potency from rat to rat now contrasts strongly with the relatively constant effect of F.M.E. or a mixture of ATP and clotting agent. This variation probably reflects the wide range in the tolerance of the rat to a clotting agent.

TABLE IV.—*Showing Changes in Adenosine Equivalent and Phosphorus Distribution in Tissue Extracts Standing at Room Temperature. Results in mg./100 ml. Saline Extract* (1 : 2).

Tissue.	Time after death (mins.).	Inorganic P.	7 min. acid-labile P.	Adenosine equivalent.	Lethal dose ml./100 g. rat.
Muscle	15	16·9	4·5	40	0·5
„	60	26·8	4·4	20	0·7
„	120	30·2	1·8	7	0·8
„	180	34·3	1·3	6	1·0
60° C. for 15′	25	30·2	1·2	2	1·5
Testis	10	1·2	2·0	18	0·4
„	180	13·2	1·1	2	0·8
Lung	10	3·9	0·6	15	0·15
„	180	6·1	0·2	2	0·25

Mechanism of Action.

If it be assumed that death following F.M.E. is due to thrombosis in vital parts of the circulation, then it would seem likely that ATP acts by virtue of its being a powerful inhibitor of cardiovascular tone. The resulting fall in circulation rate and pressure might well provide the conditions necessary for the induction of thrombosis by an otherwise inadequate amount of thromboplastin. If this is so, then other circulatory depressor agents should produce a similar effect. Indeed, acetylcholine did so, and 20 μg. + 5 units of thrombin produces a response similar, in its early stages at least, to that produced by 0·5 mg. ATP + 5 units of thrombin. Unfortunately for the hypothesis other circulatory depressants are not similarly active. The combination of thrombin with histamine (up to 3·5 mg.) is no more active than histamine alone. Moreover the degree of thrombosis in lung vessels is unaffected and, in the doses used, minimal. Still more interesting is the fact that adenosine has a relatively feeble action. Now the depressor activity of adenosine in the rat is about twice that of ATP (weight for weight). Nevertheless only when very large doses (7·5 mg. upwards) in combination with thrombin are used is there any respiratory depression comparable with that produced by 0·5 mg. ATP + 5 units of thrombin. Even at the high dosage level of 7·5 mg. adenosine + 10 units thrombin, survival is the rule. Such a dose is approaching the intravenous lethal dose of adenosine itself (10–15 mg.), so that whilst adenosine cannot be considered entirely inactive, relative to ATP it is so. Incidentally this finding makes it clear that death from ATP + clotting agent is in no way due to summation of the cardiovascular effects of the two agents.

We have seen that interference with the lung circulation by thrombosis is not in itself sufficient to account for death. We were thus led to consider whether death is primarily due to respiratory failure, and if so, whether thrombosis in the lung vessels is an essential factor in the mechanism or merely an incidental

finding. Both ATP and acetylcholine are respiratory depressants, and this action is powerfully reinforced when they are combined with thrombin. On the other hand, adenosine and histamine have no significant respiratory action, either alone or combined with thrombin. Adenosine, however, has a similar depressant action to ATP in the Mg-treated rat (see Green and Stoner, 1944), and under these conditions, when combined with thrombin, becomes nearly as active as does ATP. It seemed likely therefore that the main effect of the clotting agent is on the respiratory system. We have some evidence in the rat that ATP inhibits respiration partly by a direct action on the medullary centre and partly by stimulation of vagal afferent fibres at the periphery. It therefore seemed possible that the clotting agent in some way strongly reinforces one or both of these actions. That such a mechanism is involved is revealed by the finding that after section of the vagi the rat easily withstands previously fatal doses of ATP + thrombin. There is now no prolonged respiratory inhibition; in fact the period of apnoea is somewhat less than that seen with ATP alone in the intact animal. The prolongation of the ATP-induced bradycardia is also abolished. Intravenous atropine diminishes and sometimes abolishes the effect, but the results are more erratic and further investigation is needed. We have previously found that stimulation of the central end of the cut vagus enhances the respiratory effects of ATP, so that it is highly probable that clotting agents directly or indirectly act likewise. Possibly also their accentuation of the cardiovascular effects of ATP is similarly mediated reflexly through the vagus.

This powerful increase in vagal afferent stimulation in the presence of ATP is probably due to the much enhanced degree of thrombosis in the lungs, and it remains to account for the manner in which ATP brings this about. The number of vessels involved is certainly no less after section of the vagi, so that the accentuation of thrombosis is not due to the prolonged respiratory inhibition. It is more likely that it is a result of the constrictor action of ATP on the pulmonary vascular bed, which Gaddum and Holtz (1933) demonstrated in the cat. We have confirmed that this effect is very powerful. The resulting slowing of the flow in the peripheral vessels might well provide the additional factor necessary to account for the great accentuation of thrombosis by the clotting agent. Adenosine, according to Gaddum and Holtz (1933), has only 1/15 the constrictor activity of ATP, a degree of activity which correlates well with the respective potencies of the two substances when combined with clotting agent. Even this hypothesis is complicated by the fact that histamine constricts pulmonary vessels (Dale and Laidlaw, 1910), but this discrepancy is possibly explained by differences in the type of lung vessels involved.

It would seem that the rapid death produced by intravenous clotting agent + ATP is due to the prolonged respiratory inhibition. Delayed death may well be due to permanent damage to the medullary centres brought about by prolonged anoxaemia.

We have not yet analysed the rather similar effect of ATP and acetylcholine, but it would seem likely that a similar mechanism is involved.

Intravenous Effects of Other Tissue Extracts.

All the fresh saline extracts prepared from other organs, with the exception of liver, are lethal at doses of 0·5 ml. or less (Table II). Since the reaction of the rat is essentially similar in all cases to that obtained with F.M.E., and since

moreover their potency falls slowly on standing and rapidly on heating, and they are antagonized by prophylactic heparin, there is an *a priori* case for suggesting that the mode of action of all these tissue extracts is essentially similar. All contain thromboplastin and all probably some ATP (Tables II and III), which, like thromboplastin, is rapidly inactivated on heating and, unlike thromboplastin, gradually breaks down at room temperature (Table IV). Their thromboplastin contents vary greatly, and this would seem to be the determining factor in the variations in lethal potency. The richest source, lung, yields the most potent extract, whilst liver extract, in the range of doses used, is relatively inert. The potency of all is strongly augmented when mixed with ATP. The increase in potency is directly proportional to their thromboplastin content, liver extract having only slight activity. It is obvious from the figures given (Table III) that in none of these does the ATP content approach that of F.M.E. On the other hand, their adenosine content is half or more that of F.M.E., and if the valid assumption is made that all or most of the adenosine is phosphorylated, it can be postulated from all our findings that such compounds (or their deaminated derivatives) in combination with thromboplastin could account for most or all of the intravenous toxicity of the extracts.

Possible Role of Clotting Factors in Ischaemic Shock.

Since this work owes its origin to a more general study of ATP metabolism in shock, a few observations bearing directly on the role of clotting factors in relation to tissue injury are relevant here.

The first question to decide was whether the coagulant-ATP effect can be elicited if ATP is injected by a route other than the intravenous. With intraperitoneal doses approaching the lethal (45 mg.), intravenous O.M.E. (0·2 ml.) produces the typical response and fatal effect of giving the combined agents intravenously. The effect is obtained up to at least 1 hr. after ATP is injected. With smaller doses of ATP (25 mg.) the effect is relatively slight. There is little doubt, however, that ATP absorbed in adequate amounts from the tissues is rapidly fatal when there is an otherwise innocuous amount of coagulant in the circulating blood.

Another relevant question was whether injured tissue shows any alteration in its content of clotting factor. In this a lead was given by the work of Quastel and Racker (1941), who found an increase in the thromboplastin content of the rat hind limb following a period of ischaemia. This finding has been confirmed and amplified considerably, and it has been found that extracts from muscle after a period of ischaemia which would be fatal show three times as much thromboplastic activity as normal muscle extracts (Stoner and Green, 1947). This difference has received biological confirmation in that F.M.E. (heated at 50° C. for 15 min.) and O.M.E. prepared from similarly treated muscle is more potent combined with ATP than control extracts from the opposite limb. Whether any thromboplastin leaves the limb after release of the occlusion is not known, for the problem is technically highly complicated.

DISCUSSION.

This investigation raises issues of both practical and theoretical interest. On the practical side it demonstrates that the intravenous toxicity of fresh muscle extract can be accounted for by the combined action of thromboplastin

and adenosine triphosphate. We have also shown that such a combination of clotting agent and nucleotide is not peculiar to muscle extract, but is probably responsible for the intravenous toxicity of fresh saline extracts from many organs, and maybe all, in varying degrees. Here we may note that liver extracts are less toxic than most because of their deficiency in thromboplastin. Nor is it a species peculiarity, for all the animals tested (rabbit, cat, rat and mouse) were sensitive not only to fresh muscle extract but to combinations of various clotting agents and adenosine triphosphate. There are, however, indications that the thromboplastin component is more effective in the homologous species, for rabbit muscle extract, in combination with ATP, was not nearly so potent in the rat as was rat extract of equal thromboplastic activity. These species differences have not been worked out in detail, but attention is called to them since such differences are revealed by *in vitro* investigations (Quick, 1942).

As a result of this work the mode of action of these toxic agents in fresh tissue extracts becomes reasonably clear. ATP and all the nucleotides we have tested are, in suitable dosage, respiratory depressants. It would seem that the effect of the clotting agent is to reinforce or potentiate this action by inducing peripheral stimulation of the vagus. This peripheral stimulation may be the direct result of the great enhancement of the clotting process in lung vessels produced by clotting agent combined with ATP. There is no *in vitro* evidence that ATP has a direct action on the clotting mechanism, and it seems probable that it accentuates thrombosis by virtue of its powerful constrictor action on lung vessels. The nature of the clotting agent does not matter, and it is the clotting process itself rather than the exciting agent which apparently produces vagal stimulation. We have found that rather similar effects to those produced by ATP + thrombin can be obtained with ATP + fat, which incidentally results in a great reduction in the lethal dose of fat. Shaw Dunn (1920) has shown that small emboli in the pulmonary arterioles produce alterations in respiratory rhythm which are abolished by section of the vagi. It is likely then that the presence of particulate matter in the lung vessels in itself produces vagal stimulation. The conclusion emerges that death from intravenous tissue extract is only indirectly due to thrombosis in lung vessels, and not, as might have been expected, to a lethal interruption of the lung circulation.

There can be little doubt that a mechanism similar to that described is involved in the action of other fresh tissue extracts, though the relative importance of thromboplastin and ATP (and/or nucleotides) may vary greatly in different tissues. The cardiovascular effects of ATP and fresh tissue extracts are also accentuated by clotting agents, but these effects probably play a relatively minor part in the intravenous lethal action of the tissue extract. Thrombin has little or no reinforcing effect on the toxicity of the powerful depressor agents, adenosine and histamine, which shows, incidentally, that a fall in systemic blood pressure does not predispose to thrombosis in the lung vessels.

It may be asked how far these findings can be applied to the mode of action of intravenous clotting agent alone. Obviously the same mechanism is involved in some degree, for large doses in themselves are respiratory depressants, and a lethal dose of thrombin becomes non-lethal after section of the vagi. Death in the intact animal is therefore not necessarily the direct result of vascular occlusion in vital areas, but in part at least a result of respiratory failure of central origin. The work of Mylon, Winternitz, Katzenstein and de Sütö-Nagy (1942) points in the same direction. Using acetone-dried testis as a source of thromboplastin

they found that section of the cervical cord in the dog rendered a previously lethal dose practically innocuous, though clot formation in the lung occurred as usual. This, together with other findings, led to the conclusion that the general and more serious effects of thromboplastin were mediated through the central nervous system. In the light of our work their described effects were probably not due entirely to thromboplastin for the injected substance probably contained some nucleotide, but in any case, their findings harmonize well with those we have described.

Freund (1920) first gave the name "fruhgift" to the toxicity of fresh defibrinated blood and serum when injected intravenously in the rabbit, a toxicity which disappears after 24 hours' storage. It was suggested by Zipf (1931) that "fruhgift" might be identical with adenylic acid. Kashibawara (1934) showed that "fruhgift" was produced in the process of coagulation, and that its effects could be antagonized by previous injection of sodium citrate. He concluded that a clotting substance, and not adenylic acid, was the responsible agent in "fruhgift." In view of our results the possibility that serum nucleotides potentiate the effect of thrombin in fresh serum ought to be considered.

It should be noted that our findings have probably no bearing on the nature of the toxic factor developing in plasma or serum after prolonged storage. This substance does not appear to be concerned in the clotting mechanism, although, like clotting agents, its effects are mainly due to reflex action through the vagus (Gilding and Nutt, 1944).

A knowledge of the nature and mechanism of the main toxic components in fresh tissue extracts is obviously of crucial importance in biological research. Otherwise purely non-specific toxic effects will be, and have frequently been, ascribed to a specific action of the tissue. Moreover, and probably still more important, measures for destroying or antagonizing such non-specific effects may reveal acute or delayed specific effects which otherwise are completely hidden. The present work shows that this can be achieved in large measure either by previous heparinization of the animal or by heating the extract to 60° C. for 15 min., whichever best suits the experimental approach. Heparinization does not affect the response of the animal to ATP or its derivatives. These compounds are not, however, in sufficient concentration in a lethal dose of fresh tissue extract to produce by themselves a more than transient reaction.

These considerations only apply directly to unheated watery extracts. With other methods of extraction the question of how far the method would remove or destroy nucleotides and clotting agents would need to be considered individually. For instance, acetone treatment of very fresh tissue will preserve much of its ATP content, but in the case of muscle, most of the thromboplastin is thereby removed, and the activity of the saline extract of the dried tissue is solely that of its nucleotide content.

The present work was the outcome of a study of the mechanism of ATP shock and its bearing on the shock state in general. Now muscle is a rich source of ATP, and its thromboplastin content increases greatly after a period of ischaemia. It is tempting, therefore, to hypothesize that these two factors play a combined role in the genesis of ischaemic and related forms of shock. Such a temptation should be resisted, for we still lack conclusive evidence that ATP or related compounds are released from injured tissues *in sufficient quantity* to induce a severe general reaction. Moreover we do not yet possess a satisfactory technical approach to the problem of whether clotting agents are released in

abnormal amounts. There are numerous recorded observations on alterations in blood coagulability following tissue injury, but they are not consistent. If speculation be allowed, perhaps the most likely is that the described effects may be related to the disorders of the lung which are the most frequent of the delayed clinical complications of severe tissue injury. About 15 per cent of the rats which apparently recovered from the early effects of combined clotting agent and ATP died later, and all showed broncho-pneumonic changes. Whether these and other findings reported here will prove to have a bearing on clinical problems in man remains to be seen.

SUMMARY.

The lethal effect of an intravenous dose of a fresh saline muscle extract is due to the combined action of thromboplastin and adenosine triphosphate and/or related phosphorylated compounds.

The diminution in potency on standing is due to the enzymatic destruction of adenosine triphosphate and related active compounds. Heating at 60° C. for 15 min. removes practically all the toxicity by inactivating both adenosine triphosphate and thromboplastin.

Previous heparinization of the animal by antagonizing the *in vivo* action of thromboplastin, though not of adenosine triphosphate, makes it tolerant to an otherwise lethal dose of extract.

The toxicity of many, and maybe all, fresh saline tissue extracts may be accounted for by similar factors, and is antagonized by similar measures.

Identical effects to those obtained with tissue extracts are obtained by combining adenosine triphosphate and any source of intravascular clotting substance.

Adenosine triphosphate greatly increases the clotting action of thrombin in the lung vessels. This may be due to the powerful constrictor action of adenosine triphosphate on the pulmonary vessels.

Death produced by the combined action of adenosine triphosphate and thrombin is primarily due to respiratory failure. There is evidence that the enhanced clotting process in the lung vessels induces vagal stimulation, which potentiates the inhibitory action of adenosine triphosphate on the respiratory centre.

The possibility that the combined action of adenosine triphosphate and clotting agent plays a part in the mechanism of ischaemic shock is discussed.

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STUDIES ON STAPHYLOCOCCUS MUTATION: CHARACTERISTICS OF THE "G" (GONIDIAL) VARIANT AND FACTORS CONCERNED IN ITS PRODUCTION.

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A DESCRIPTION of a small colony variant of *Shigella shigae* was given by Hadley, Delves and Klimek (1931), who suggested that it formed part of a normal life cycle of the organism. Similar "G" or gonidial mutants were obtained from *Staphylococcus aureus* by growth in media containing lithium chloride (Hoffstadt and Youmans, 1932; Swingle, 1935), by animal inoculation (Hoffstadt and Youmans, 1932), and by growth in peptone water containing barium chloride or barium nitrite (Youmans and Delves, 1942).

The hypothesis that the "G" variant represented a stage in the normal life cycle of the staphylococcus was discarded for lack of any corroborative experimental evidence in favour of the view that the small colony was composed of cocci whose metabolic activities were less than those of the parent cells. To account for this metabolic difference Swingle (1935) suggested that an occasional cell became damaged, and that the resultant alteration was subsequently transmitted through successive cell generations. Destruction of cellular enzymes or their inhibition by the chemical compounds used in the production of the small variant (Youmans and Delves, 1942; Colwell, 1946) or a decrease in cell permeability (Youmans and Delves, 1942) were also advanced as possible causes of this lowered metabolism.

Reports of an increased resistance of the "G" variant to penicillin (Schnitzer,

Carmagni and Buck, 1943) and also to certain dyes (Dubos, 1945) bear the implication that mutation of a staphylococcus to this form may affect the course of an infection, especially in response to chemotherapy. This calls for a more precise knowledge of how the variant arises and how it differs from the parent strain in respect of metabolic activity. The first stage of an investigation directed to this end forms the subject of the present paper.

PRODUCTION OF A STABLE "G" VARIANT.

Nutrient agar plates inoculated with staphylococcal pus often show a number of small unpigmented colonies among the normal large ones. Although these resemble the description of the "G" colony type, they either revert to the parent type or again give a mixture of large and small colonies when explanted. It has never proved possible by simple colonial selection to obtain a small colony strain that bred true. The production of a stable "G" variant by growth in lithium chloride has been reported (Hoffstadt and Youmans, 1932; Swingle, 1935). Efforts to confirm this were generally unsuccessful; small colonies were occasionally obtained, but they reverted to the parent form, after, at the most, two successive platings on medium devoid of lithium chloride. The following descriptions are not concerned with this type of colony, which may be referred to as a temporary "G" variant, but with a stabilized variant that retains its small colony form throughout successive cultures on nutrient agar.

Growth of the staphylococcus in the presence of barium chloride.

Barium chloride was added to 1 per cent peptone in distilled water so that a final concentration of M/15 barium chloride was obtained. (Certain brands of peptone gave a slight haziness when barium chloride was added, due to the precipitation of insoluble phosphates, so that it was necessary to filter such media before use.) The medium was tubed in 5 c.c. quantities, inoculated with staphylococci and incubated at 37°C. No growth was usually apparent in the tubes for the first five to seven days, after which period a slight turbidity appeared. Explants made from these tubes on to nutrient agar plates gave from 90 to 100 per cent of small colony variants. The stability of such variants was ascertained by successive explants on nutrient agar plates.

Although this method gave positive results in the majority of experiments it was not infallible, and it soon became apparent that the size of the original inoculum influenced the result. Tubes of 5 c.c. quantities of 1 per cent peptone in distilled water containing a concentration of M/15 barium chloride were inoculated with saline suspensions of large colony staphylococci made from 18-hour nutrient agar slopes. The suspensions were enumerated in a counting chamber and adjusted to the required number of cocci. The results of a typical experiment are set out in Table I.

Inocula of $5 \times 10^4 - 5 \times 10^5$ staphylococci (Strain E) per 5 c.c. of media after a lag period of 5–10 days developed a faint haziness due to the growth of the "G" variant. Tubes receiving larger inocula usually showed turbidity overnight, and explants over a period of 14 days revealed only the large colony types, though occasionally an inoculum of 5×10^3 cocci gave a growth of the small variant. Identical results were obtained with another strain of *Staphylococcus pyogenes*, Au 35.

TABLE I.—*Growth of the Staph. E in* 1 *per cent Peptone Water Containing* $M/15$ $BaCl_2$.

Inoculum in 0·2 c.c. saline.	Incubation period.				
	17 hr.	3 days.	5 days.	7 days.	10 days.
500×10^6 cocci	L	L	L	L	L
50×10^6 ,,	L	L	L	L	L
5×10^6 ,,	NG	L	L	L	L
5×10^5 ,,	NG	NG	L	L	L
5×10^4 ,,	NG	NG	NG	S	S
5×10^3 ,,	NG	NG	NG	NG	NG

NG = No growth.
L = Growth of large colony type staphylococci.
S = Growth of " G " variants.

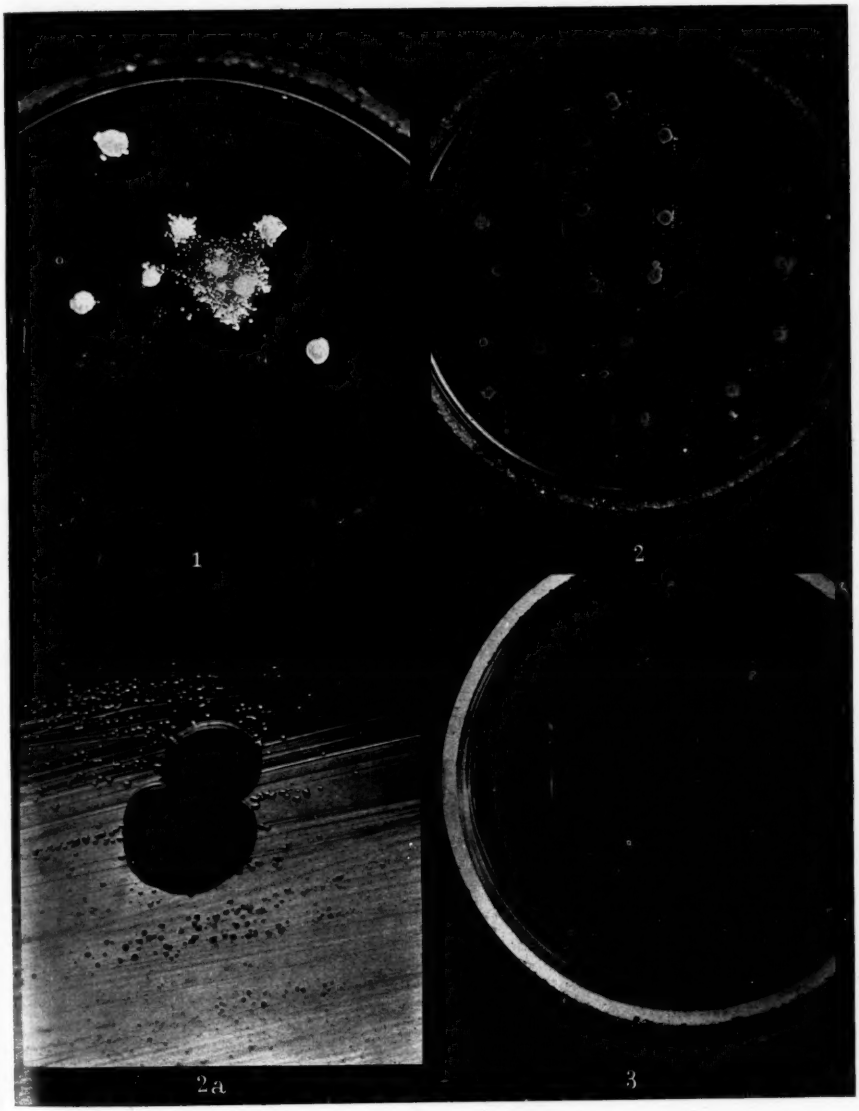
Concentrations of barium chloride greater than M/15 inhibited both the variant and the parent strain, whereas lower concentrations allowed normal growth of the parent strain, so that any selective factors operating in favour of the variant were lost. The addition of various compounds to the basic media of peptone water and barium chloride proved interesting. Using acetone extracted Lab. lemco (Youmans and Delves, 1942; Hughes, 1932) and marmite it was necessary to increase the concentration of barium chloride to achieve results comparable with those obtained by the simpler media. These substances appeared to counteract the inhibiting action of barium chloride, as also did digest broth, serum and yeast extract (prepared by extracting bakers' yeast with distilled water at 100°C. for 15 min.).

Growth of the staphylococcus on nutrient agar plates containing gentian violet.

Nutrient agar plates containing one part in a million of crystal violet or gentian violet were spread with saline suspensions of staphylococci from 18-hour agar slope cultures. The suspensions were enumerated and then diluted so that a million cocci were contained in 0·25 c.c., and this quantity was spread over each plate. In each experiment six to eight plates were put up in parallel and incubated at 37° C. After 24 hours' incubation a few normal staphylococcal colonies were seen, but with continued incubation 80–100 colonies, showing every variation in size from the large colony to pin-point size, appeared between 42 and 72 hours. A selection of about 20 colonies from each plate, which included both intermediate and small, were explanted for several generations on nutrient agar plates in order to determine the ratio of stable " G " variants, and from this it was possible to calculate the number present per 10^6 cocci initially seeded on the plate. It was found that within the limits of experimental error the stable variant appeared in a ratio of 1 per 5×10^4 cocci seeded, although the ratio in an occasional experiment might lie between 1 in 10^6 and 1 in 10^3 cocci. This ratio of one " G " variant per 5×10^4 staphylococci was in general agreement

DESCRIPTION OF PLATE.

FIG. 1.—Satellite effect obtained by growth of the staphylococcus on nutrient agar containing malachite green.
FIG. 2.—Satellism of " G " colonies around stab inoculation of the parent strain.
FIG. 2*a*.—Higher magnification of an area of the same plate.
FIG. 3.—The improvement of growth of the " G " variant in overcrowded areas of the plate.



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with the size of inoculum required to obtain the " G " variant by growth in peptone water and barium chloride media. Using gentian violet agar plates stable " G " variants were obtained from each of five *Staphylococcus pyogenes* strains tested.

Growth of the staphylococcus in the presence of other bacterial inhibitors.

The action of such diverse substances as barium chloride and crystal or gentian violet allowing growth of the variant while suppressing that of the parent coccus suggested the trial of other well known bacterial inhibitors for their ability to produce similar effects. The substances tried were thiamin, basic fuchsin, malachite green and phenol. When barium chloride or gentian violet was used the variant grew out at that concentration of the inhibitor which just inhibited the growth of the parent strain. When testing these compounds, therefore, it was essential to employ a series of plates covering a wide range of concentrations in order that one set would fall within this narrow range. Although the concentrations were made in twofold steps and ranged from those allowing full growth to concentrations well above that required to inhibit growth of the parent coccus, no small colonies were obtained. Concentrations near the limit for complete inhibition generally resulted in the appearance of a few normal staphylococcal colonies after about 36-hours' incubation, and in 72-hours these became surrounded by large numbers of small satellite colonies (Fig. 1).

These colonies had all the appearance of the " G " variant, but explanting on nutrient agar resulted in immediate reversion to the large colony form.

CHARACTERISTICS OF THE " G " VARIENT.

The " G " variant grew in unenriched media, but the rate and final mass of growth obtained was never comparable with that obtained when the parent staphylococcus was grown under identical conditions. Growth curves obtained from meat infusion broths inoculated with the variant and parent strains confirmed this, and demonstrated quite clearly the increased lag period and longer generation time of the " G " form.

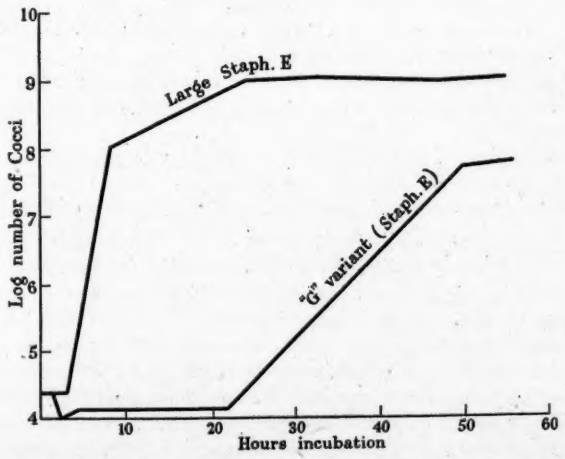

FIG. 4.—Growth of *Staphylococcus pyogenes* (Strain E) and its " G " variant in nutrient broth.

Neglecting the number of non-viable organisms resulting at each division (Wilson, 1922), and calculating the generation time from the formula—

$$G = \frac{T}{n} \text{ where } n = \frac{\log a - \log b}{\log 2} \quad \begin{cases} a = \text{Number of organisms at end of time } T. \\ b = \text{Number of organisms at beginning.} \end{cases}$$

the generation time of the parent strain growing in infusion broth was on an average 20 min., while that of the " G " variant proved to be about 120 min.

Occasionally during growth of the " G " form, cocci that had reverted to the parent form would appear. This reversion was irregular and unpredictable, but it was favoured by growth in fluid media, particularly those that allowed a fairly heavy final mass of growth. Favourable media in this respect were meat infusion broths enriched with serum, marmite or yeast extract. If the reversion occurred towards the end of the growth period, then explants on nutrient agar plates exhibited large numbers of small, with a few isolated large colonies. On the other hand, if the reversion took place during the early stages of growth, then the reverted form multiplied at a much quicker rate than the " G " form, and explanting at the end of the growth period resulted in a plate consisting almost entirely of large colonies. Plates on which a few large colonies were growing often demonstrated a striking satellite effect : the growth of the small colonies in the immediate vicinity of the large colonies was greatly enhanced. Similar satellite plates were reproduced by seeding nutrient agar plates with the small variant, and subsequently stab inoculating the parent strain into several well separated sites. The result is shown in Fig. 2 and 2*a*.

Not all the " G " variants which were obtained exhibited this phenomena of satellism when growing round the parent strain. Growth of the small colony forms on nutrient agar into which sterile culture filtrates of the parent strain had been incorporated resulted in larger colonies than did growth on the plain nutrient agar, providing that the test organism was a variant which exhibited satellism. An even greater effect was obtained by growth on plates containing yeast extract. On these plates the small variant grew with golden pigmented colonies indistinguishable from colonies of the parent strain grown on nutrient agar, except that they were perhaps a little more pigmented. When one of these pigmented colonies was transferred to unenriched nutrient agar it reverted to the small colony form.

Another feature observed during routine platings of the " G " variant was the improvement of growth on agar plates in regions where overcrowding existed, while single well isolated colonies grew extremely slowly. This contrasted sharply to findings with the normal staphylococcus where colonies develop quicker and attain larger size in areas of the plate in which separation is maximal (Fig. 3).

Morphology.

Examined microscopically the " G " variant exhibited the typical staphylococcal arrangement, and the cells tended to be larger and to show greater individual size variations than the parent coccus. They were gram positive, but in any film more frankly gram negative cocci were present than in a corresponding film of the parent strain stained by identical technique. The impression gained was that the variant belonged to what has been termed the Gram variable class.

Filterability.

Attempts to demonstrate the filter-passing ability of the variant failed completely. Filtration experiments were made with gradocol membranes of 0·76μ average pore size.

Heat resistance.

Suspensions of a million cocci per ml. broth were held at different temperatures for 30 min. Immediately at the end of the 30 min. the tubes were cooled, 30–40 c.c. of nutrient broth added and the tubes incubated at 37° C. The normal staphylococcus was killed by 30 min. exposure to temperatures between 65° and 70° C., while the small variants were less resistant and in similar conditions were killed between 50° and 60° C. Similar experiments employing saline instead of broth suspensions gave lower thermal death points between 56° and 60° C. for the normal staphylococcus and below 50° C. for the " G " variants. These findings contrast sharply with those of Hoffstadt and Youmans (1932), who found the " G " variant much more resistant to heat than the parent strain.

Penicillin resistance.

A claim that the " G " variant staphylococcus is more resistant to the action of penicillin than the parent strain has been made by some workers (Schnitzer, Carmagni and Buck, 1943). If substantiated this might have bearings both on the therapeutic use of penicillin in staphylococcal infections and on work designed to elucidate the mechanism of penicillin action. It was, therefore, deemed necessary to investigate this claim very carefully.

Tubes containing dilutions of penicillin in 1 c.c. of broth were seeded with 40,000 cocci in 0·02 c.c. saline and incubated at 37° C. for 48 hours. Readings were taken at 24 and 48 hours. The results are set out in Table II, and show that the variant is four times more sensitive to penicillin than the parent strain.

TABLE II.—*Penicillin Sensitivity of the " G " Variant Staphylococcus and Parent Strains.*

	Inoculum = 40,000 cocci.						
Organism.	8	4	2	1	0·5	0·25	*Nil.*
Staph. E (Lge.) . .	—	+	++	+++	+++	+++	+++
Staph. E, " G " variant	—	—	—	+	+	+	+
Staph. au., 18 . .	—	—	—	++	+++	+++	+++
Staph. au., 18 " G " variant .	—	—	—	—	—	+	+
Oxford Staph. . .	—	—	—	+	+++	+++	+++

Readings made after 48 hr. incubation. + = Degrees of growth.

If inocula of more than 4×10^5 cocci were used the findings were often obscured, as these large inocula sometimes contained cocci that had reverted to the parent form, which were then able to grow out in the dilutions of penicillin inhibitory to the small variant. Nevertheless, even using large inocula the small variant was never more resistant than the parent coccus, although in many tests it appeared from simple visual readings that the two organisms possessed the same sensitivity. Agar plates seeded from the tubes of such a test demonstrated that where unexpected growth had taken place in the higher concentrations of penicillin in the " G " variant series tubes, it was due entirely to large colony staphylococci.

Sugar fermentation reactions.

The variant " G " staphylococcus, unlike the parent staphylococcus possessed little saccharolytic activity. Of the sugars tested the variant produced a small amount of acid extremely slowly from glucose only.

TABLE III.—*Sugar Fermentation Reactions of " G " Variant and Parent Strain.*

Organism.	Glucose.	Lactose.	Sucrose.	Mannite.
" G " variant staphylococcus	A (48 hr.)	..	..	..
Parent staphylococcus	A (18 hr.)	A (18 hr.)	A (18 hr.)	..

A = Acid production.
Figure in brackets refers to the time acid production became apparent.

It was necessary, before finally accepting the readings of a sugar fermentation test, to ensure, by explanting on nutrient agar, that no reversion to the parent form had taken place.

These findings are in accord with those of Swingle (1935), and it is to be noted that Colwell (1946) reported the production of " G " colony variants of *Bact. coli* that possessed a lower fermentative activity than the parent *coli*.

α Toxin production and coagulase activity.

Using the sheep blood agar plate technique (Bryce and Rountree, 1936) and incubating in an atmosphere containing 10 per cent carbon dioxide, it was impossible to demonstrate the production of α toxin by " G " variants although they were derived from known toxinogenic strains.

Overnight broth cultures of mutants of staphylococcal *pyogenes* strains tested for coagulase activity by the method described by Fisk (1940) clotted human plasma but only after prolonged incubation.

Serological and phage typing of " G " variants.

A strain of *Staphylococcus pyogenes* and the " G " variant derived therefrom were kindly examined by Dr. Allison at the Central Laboratory of the Public Health Laboratory Service, who reported that " the variant and parent strain undoubtedly belonged to the same serological and phage types."

DISCUSSION.

The evidence presented suggests that the staphylococcal gonidial variant is neither a form in the life cycle of the staphylococcus, nor a form that arises as the result of some chance injury insufficient to kill the cell. It appears as a discontinuous variant in the ratio of about one in 50,000 cocci. Although the work was not carried out with single cell cultures, all the strains used were plated, a single well isolated colony picked and the process repeated many times. The plates never exhibited small colonies during these routine cultures.

To obtain the variant in pure culture it is necessary to provide conditions which, though inhibiting the parent form, allow comparatively free growth of the variant. Of the substances tested, barium chloride and gentian violet used at suitable concentrations are capable of producing these effects. The actual

mechanism of selection requires elucidation, but it may be significant that in its greater resistance to gentian violet the " G " variant approaches more the characteristics of the gram negative organisms. It has been suggested that a decrease in sensitivity to basic dyes is paralleled by a loss of gram positiveness (Stearn, 1927). Preliminary experiments with dyes indicated that the variant and parent cocci had slightly different iso-electric potentials, the " G " variant in this respect approximating to groups of organisms classified as gram variable, e.g. Corynebacteria. This relatively slight difference in iso-electric potentials may also account for the concentration of gentian violet which is effective in selecting the variant, being only slightly greater than that which totally inhibits the parent coccus, and for the impossibility of attaining this narrow range when using more basic dyes such as malachite green, etc.

The cultural characteristics and lower fermentative ability of the variant definitely substantiates claims of a lowered metabolic activity made by previous investigators. Satellism exhibited by certain of the " G " variants when growing round a colony of the parent strain suggests that the former is nutritionally more exacting than the latter, requiring some growth factor present in minimal amounts in the nutrient agar plate, but which is synthesized and distributed into the surrounding media by the parent strain during growth. If this factor could be supplied in optimal quantities, then the growth of the small variant should be indistinguishable from that of the parent strain. This is in fact seen when such a variant is grown on a nutrient agar plate incorporating yeast extract. The " G " variants which do not show satellism must be dependent upon a different compound not synthesized by the parent strain or possibly a second additional growth factor.

It is extremely interesting that an organism should throw off with regular sequence a mutant less equipped for existence than itself, and the study of this different metabolism should yield valuable information about the metabolic pathways adopted by the staphylococcus. The finding of a substance that would allow growth of the " G " variant to proceed equally to that of the parent strain would indicate that this substance is a link in the chain of metabolic reactions of the staphylococcus, the synthesis of which is impaired or non-existent in the " G " variant. Lodge and Hinshelwood (1944) postulate that growth involves a complex sequence of reactions, and from any given starting-point to a given end-point a number of pathways could be used (Lodge and Hinshelwood, 1944; Hinshelwood, 1944). The staphylococcus may have two mechanisms, 1*a* and 2*a*, both capable of producing substance A, 2*a* being more efficient and used under normal growth conditions. Under selective conditions the 2*a* mechanism may be suppressed and finally lost so that the " G " variant arises, possessing only mechanism 1*a*. Consequently the growth of the " G " variant would be much slower than the parent strain with its efficient 2*a* mechanism, but could be stimulated by the addition of substance A to the culture media.

The more exacting nutritional requirements of the " G " variant accounts for its relatively better growth on an agar plate where overcrowding exists. In these areas the large number of cocci build up a local concentration of the essential nutrient. It is a phenomenon not confined to this variant, but is noticeable whenever attempts are made to cultivate rather exacting organisms on unenriched media, and for identical reasons large inocula are necessary for growth to take place in deficient media.

Attempts to elucidate the nature of the nutritional differences between the

"G" variants and parent staphylococci and to define the growth promoting substances involved are in progress.

SUMMARY.

1. Small colony "G" (gonidial) variants that remain stable after successive generations on nutrient agar plates were obtained by growth of staphylococcus in peptone water containing barium chloride and on nutrient agar plates containing gentian violet.

2. This "G" form appears to be a discontinuous variant arising once in approximately 50,000 cocci.

3. Its metabolic activities as estimated by growth rate and sugar fermentations are much lower than the parent strain.

4. "G" variants have less resistance to heat and penicillin than the strains from which they are derived.

5. The variant is feebly coagulase positive, but produces no α toxin.

6. The possibility that the variant is nutritionally more exacting than the parent strain is discussed.

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The author also wishes to express his indebtedness to Professor Wilson Smith for constant advice and criticism, and to Dr. Allison for his report on serological and phage typing.

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INHIBITION OF GROWTH OF *BACT. TYPHOSUM* BY METHYL DERIVATIVES OF INDOLE AND TRYPTOPHAN.

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A LARGE number of substances have now been designed (Roblin, 1946) on the model of essential metabolites, to act as bacterial inhibitors by competition with the essential metabolites. This principle was made familiar by Woods (1940) in the case of sulphanilamide and *p*-aminobenzoic acid (*cf.* Fildes, 1940).

It is largely accepted that bacteria and other cells in the course of growth necessarily carry out a number of syntheses, one of which is the series which includes tryptophan. From the work of several authors in different fields (Snell, Tatum, Bonner, Beadle, Fildes) the generalization has developed, and is here accepted, that the tryptophan series, in the case of most cells, involves the reaction of anthranilic acid with an unknown substance to form indole, and this in turn with serine to form tryptophan, which is then further utilized presumably for protein synthesis. Inhibitors designed on the model of indole or tryptophan may compete with either of these essential metabolites, and so block the synthesis above indole and serine but below tryptophan, or above tryptophan. The position of the block is deduced by the behaviour of an organism of known characters in the presence of the inhibitors.

Fildes (1941, 1945) has already shown that indoleacrylate inhibits by preventing the coupling of serine and indole, and so preventing the synthesis of tryptophan. The present paper deals with methyl-indoles and -tryptophans, the target essential metabolites being, again, indole and tryptophan.

EXPERIMENTAL DETAILS.

The test organism used was *Bact. typhosum* 3390. Technical details have been described by Fildes (1945).

It may be recalled that 3390 is an organism which has a nutritional block immediately below indole. Thus, it cannot grow until indole has been supplied, and since growth then proceeds normally it is deduced that it can synthesize serine, which is absent from the medium, and couple it with indole to form tryptophan. It can also make use of tryptophan for further purposes of growth or reproduction.

The methyl-indoles and methyl-tryptophans used were all synthesized by unambiguous methods which will be described elsewhere (Rydon, in the press).

NOMENCLATURE.

The methyl derivatives are named in accordance with the system shown in Fig. 1, which is now generally accepted.

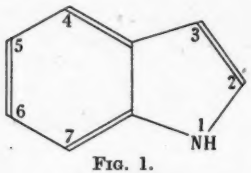


FIG. 1.

THE METHYL-INDOLES (MI) AND METHYL-TRYPTOPHANS (MT) AS GROWTH FACTORS.

Preliminary tests were carried out to show that the introduction of a methyl group into indole and tryptophan was sufficient to destroy their biological effect as growth factors.

The seven methyl-indoles and the five methyl-tryptophans had no action in causing growth of 3390 in circumstances in which indole or tryptophan were active at 10^{-6} M concentrations or lower.

METHYL-INDOLES AND METHYL-TRYPTOPHANS AS BACTERIAL INHIBITORS.

Methyl-indoles.—Inhibitory action was tested in the presence of indole as the necessary growth factor. A single concentration of inhibitor was used and inhibition judged by the time lag in growth. Exp. 1 shows that 1-, 2- or 3-MI gave no demonstrable inhibition, but that 4-, 5-, 6-, 7-MI were all inhibitory in the order 4>6>7>5.

Methyl-tryptophans.—Similar orientating tests with the methyl-tryptophans showed that 2 MT had little, if any, inhibitory action (Exp. 2), while the other preparations all inhibited in the order 4>5>6>7.

EXP. 1.—*Inhibition by Methyl-indoles.*

Flask.	Indole ·00002 M.	MI ·0002 M.	Growth in hours or days. 24	46	3
1	0	0	0	0	0
2	+	0	+++	+++	+++
3	+	1-MI	+++	+++	+++
4	+	2-MI	+++	+++	+++
5	+	3-MI	+++	+++	+++
6	+	4-MI	0	0	+
7	+	5-MI	tr.	++	+++
8	+	6-MI	0	tr.	+++
9	+	7-MI	0	+	+++

Plus signs are roughly proportional to mass of growth. tr. = trace.

EXP. 2.—*Inhibition by Methyl-tryptophans.*

Flask.	Indole ·00002 M.	MT ·0002 M.	Growth in hours or days. 21	28	2	3	6
1	0	0	0	0	0	0	0
2	+	0	++	+++	+++	+++	+++
3	+	2-MT	+	++	+++	+++	+++
4	+	4-MT	0	0	0	0	0
5	+	5-MT	0	0	tr.	+++	+++
6	+	6-MT	0	0	+	+++	+++
7	+	7-MT	0	+	+++	+++	+++

The inhibitory action of 5-MT has been recorded by Anderson (1945), who concluded that it " interferes in some manner with either the synthesis or utilization of tryptophan."

DATA FOR ANALYSIS OF INHIBITORY ACTION.

With the object of analysing the mechanism of these inhibitions, quantitative experiments were carried out with the two most inhibitory compounds 4-MI and 4-MT.

In experiments of this sort it is assumed that inhibitors modelled on essential metabolites exert their action by interfering with the normal synthetic processes in which the essential metabolites are concerned. This interference may be exerted in at least two ways, namely, (*a*) by preventing or delaying synthesis of an essential metabolite, or (*b*) by delaying or preventing its further use as a component in the synthesis of a more complex compound. In both cases the observed result is delay or prevention of growth in a given time. It is generally accepted that in case (*a*) the addition of the essential metabolite, of which the synthesis is delayed, will entirely counteract the inhibition in minimal concentrations and without relation to the concentration of inhibitor present. In case (*b*) it is argued that the inhibitor is in competition with the essential metabolite for the use of its enzyme, and thus that there will be a quantitative relation between the two substances, so that the same degree of inhibition will be observed whenever the metabolite/inhibitor ratio attains a certain constant figure.

In practical work it may be doubted whether tests can be carried out under sufficient control in an ever-changing biological system to give results which can be expressed usefully in numerical terms. Certainly, in much of the literature on metabolite/inhibitor ratios the basic requirements for this purpose seem lacking. Thus:

(*a*) The enzyme which an inhibitor is supposed to be affecting has two substrates, not one, and a quantitative relationship between one of them and the inhibitor must clearly be influenced by the concentration of the other. In most cases in which metabolite/inhibitor ratios have been recorded, the second substrate has even been unknown. In the tryptophan series, however, the substrates of the enzyme which synthesizes tryptophan are known to be indole and serine. In establishing metabolite/inhibitor ratios it is thus possible to ensure that one substrate is tested in excess of the other. Only in this case can a significant quantitative result be expected.

(*b*) If an essential metabolite is capable of being synthesized by the bacterium, no ratio can be determined on the basis of added concentrations. Thus, while an indole/inhibitor ratio might be determined by experiments with 3390, a serine/inhibitor ratio could not, because this organism can synthesize serine.

(*c*) Neither would it be reasonable to expect to find a demonstrable ratio between an inhibitor and an essential metabolite which is subject to attack in other ways by the bacterium, as is serine by deamination by *Bact. typhosum.*

(*d*) The more prolonged the time interval before the ratio is recorded the greater are the chances of fixation of the essential metabolite. In infinite time, in whatever concentration of inhibitor, fixation of the metabolite must at last occur and cause growth.

(*e*) The end-point taken by different authors has been so variable as to render any comparison impossible. Some, for instance, take a ratio at which growth

with and without inhibitor is the same. In the present work no such ratio has been found. Even minimal concentrations of inhibitor have some demonstrable effect, as would be expected. Others have taken the ratio at which visible growth does not take place in a convenient time, i.e., at which complete inhibition persists during observation. An end-point of this type has been used in the present work, but a longer period of observation often changes the ratio, and in practice it is not always found that a ratio is constant. Quite apart from the error inherent in most biological experiments, it seems probable that the absolute concentrations of the reactants have some bearing, especially, for instance, when one of them is near the concentration which limits its biological effect.

The occasions on which inhibitor/metabolite relationships can be expressed quantitatively by a numerical ratio are clearly few, in the present state of knowledge, but nevertheless the existence of competition can be determined in a less quantitative but more satisfactory manner. For instance, the inhibitor may be titrated in the presence of excess metabolite and the metabolite with excess inhibitor. When increase of inhibitor is found to result in increased inhibition and increase of metabolite in decreased inhibition, it may be concluded that these two substances are probably involved in a competition which delays the synthesis of a third substance. But proof of this depends upon being able to show that the third substance—the inhibited product—*counteracts the inhibition non-competitively.*

THE ACTION OF 4-METHYL-INDOLE.

Indole, Serine/4-MI Relations.

The range of dilutions at which these relations can be tested is limited by solubility, toxicity and activity considerations. The highest convenient concentration of indole is M/2000 and the lowest which will give approximately full growth is about M/125,000. Serine is highly soluble, but is inhibitory above M/1000. The highest concentration of 4-MI which can be tested conveniently is M/2000. Thus the most extreme I/4-MI ratio which can be tested is of the order of 1/60.

Since the organism used (3390) is capable of synthesizing serine and also is able to deaminate it, it would be impossible to show any constant quantitative relationship between it and the inhibitor, even if a quantitative relationship

EXP. 3.—*Indole, Serine/4-MI Relationships.*

Indole constant at $12{\cdot}5 \times 10^{-6}$ M.

Flask.	4-MI $\times 10^{-6}$ M.	Serine 12·5 $\times 10^{-6}$ M.	Growth in hours or days. 24	27	48	3	5	R. I/MI.
1	0	0	++	+++	+++	+++	++	—
2	500	0	0	0	0	0	0	1/40
3	250	0	0	0	0	tr.	+	1/20
4	125	0	0	0	tr.	++	++	1/10
5	62·5	0	0	0	+	+++	+++	1/5
6	0	+	++	+++	+++	+++	+++	—
7	500	+	0	0	0	0	0	1/40
8	250	+	0	0	0	tr.	+	1/20
9	125	+	0	0	+	++	++	1/10
10	62·5	+	0	tr.	++	+++	+++	1/5

existed. In fact, when 3390 is grown with inhibitor and indole, with and without serine, no considerable difference is found in the rates of growth (Exp. 3). This lack of activity of serine is contrasted with its action upon inhibition by indoleacrylate. Fildes (1945) showed that serine largely counteracted the inhibition by indoleacrylate, and argued that the inhibitor exerted its action both on indole and serine. In the present case it should follow that 4-MI exerts its action on indole alone.

The relationship of indole to 4-MI can be determined by titrating one in the presence of constant concentrations of the other. In all cases it is found that increased concentrations of indole reduce inhibition, so that at an I/4-MI ratio as high as 1/1 growth may even occur on the first day. Similarly, the inhibition increases as the concentration of 4-MI is increased. The following list shows the actual inhibition of growth observed in ten different experiments with different ratios and different absolute concentrations :

Ratio I/4-MI ≡ 1/1 = growth in 1 day (2 exps.).
1/5 = ,, ,, 3 days (1 exp.).
1/10 = ,, ,, 3 ,, (1 ,,).
1/16 = ,, ,, 5 ,, (1 ,,).
1/16 = no growth in 6, 7 days (2 exps.).
1/40 = ,, ,, ,, 5, 7 ,, (3 ,,).

From these results it may be concluded that complete inhibition is likely to be found during several days at a ratio of 1/30. Since indole is not synthesized and serine does not affect the result, it is probable that this figure has some significance.

T/4-*MI Relations.*

When tryptophan is used as the growth factor in place of indole, the MI series exhibits little or no inhibition. Exp. 4 shows the effect with 4-MI. It is seen that only a little inhibition occurs in the early stages which, in fact, is not uncommon with M/2000 indole itself.

EXP. 4.—*Action of 4-MI in Presence of Tryptophan.*

Flask.	Indole $\times 10^{-6}$ M.	Tryptophan $\times 10^{-6}$ M.	4-MI 5×10^{-4} M.	Growth in hours or days. 19*	24	2	4
1	—	0	0	0	0	0	0
2	—	31	0	5	+++	+++	+++
3	—	16	0	5	+++	+++	+++
4	—	8	0	3·2	++	++	++
5	—	31	+	2·4	+++	+++	+++
6	—	16	+	2·5	++	+++	+++
7	—	8	+	2·4	++	++	++
8	16	—	+	4	0	0	0
9	16	—	0	0	++	+++	+++

* Bact./cc. $\times 10^8$, converted from Hilger " Spekker " absorptiometer readings.

Thus, 4-MI has been shown to behave towards indole in a manner consistent with competition, and to exert no inhibition in the presence of tryptophan. It

may properly be concluded that it introduces a block in the synthesis of tryptophan between indole and tryptophan. Further, since its action is unaffected by serine, it may be concluded that the block is due to a competition with indole for the indole-receptive area of the enzyme only.

THE ACTION OF 4-METHYL-TRYPTOPHAN.

4-MT as sodium salt can be used in concentrations as high as M/150 final and thus a ratio I/4-MT ≡ 1/800 may be attained.

The action of serine on growth in the presence of indole and 4-MT is quite different from its action in the presence of 4-MI.

Exp. 5 shows the effect of titrating 4-MT with constant indole and with and without serine. It is seen that a higher concentration of 4-MT is required to produce the same effect with serine than without, i.e. 1/64 as compared with 1/1. Serine thus reduces the inhibition even though it is still marked.

EXP. 5.—*Indole, Serine/4-MT Relationships.*

Indole constant at 52×10^{-6} M.

Flask.	Serine 52×10^{-6} M.	4-MT $\times 10^{-6}$ M.	Growth in hours or days. 22	27	46	64	5	R. I/4-MT.
1	0	0	++	+++	+++	+++	+++	—
2	0	416	0	0	0	0	0	1/8
3	0	208	0	0	0	0	0	1/4
4	0	104	0	0	0	0	0	1/2
5	0	52	0	0	0	0	0	1/1
6	0	26	0	0	0	tr.	++	2/1
7	+	0	++	+++	+++	+++	+++	—
8	+	6667	0	0	0	0	0	1/128
9	+	3333	0	0	0	0	0	1/64
10	+	1667	0	0	0	tr.	tr.	1/32
11	+	833	0	0	0	tr.	+	1/16
12	+	416	0	0	0	+	++	1/8
13	+	208	0	0	tr	++	+++	1/4

When, however, indole and serine are titrated against constant 4-MT (Exp. 6) there is no evidence that increased concentration of either reduces the inhibition to any marked degree and, therefore, no evidence of competition between 4-MT and the essential metabolites.

Tryptophan also has an effect on 4-MT inhibition quite different from its effect on 4-MI. Tryptophan counteracts the inhibition by 4-MI non-competitively, i.e. over the whole range of its biological activity tryptophan is equally effective whatever the concentration of 4-MI.

In the case of 4-MT, however, tryptophan counteracts the inhibition more effectively as its concentration rises (Exp. 7) or, alternatively, the inhibitor becomes more effective as its concentration rises (Exp. 8).

These are two of the experimental results which permit a tentative conclusion that tryptophan and 4-MT are in competition, but proof of this conclusion is lacking because the product of the inhibited synthesis is unknown, and cannot be used to show that it counteracts the inhibition by 4-MT *non-competitively*.

EXP. 6.—*Indole, Serine/4-MT Relationships.*

4-MT constant at 833 × 10^{-6} M.

Flask.	Indole × 10^{-6} M.	Serine × 10^{-6} M.	Growth in hours or days. 27	51	3	5
1	416	0	0	0	0	0
2	416	416	0	0	+	++
3	416	208	0	0	+	++
4	416	104	0	0	tr.	+
5	416	52	0	0	tr.	+
6	416	26	0	0	tr.	+
7	416	13	0	0	0	tr.
8	208	416	0	0	+	++
9	104	416	0	0	+	++
10	52	416	0	0	+	++
11	26	416	0	0	+	++
12	13	416	0	0	0	++

EXP. 7.—*Tryptophan/4-MT Relations: Titration of Tryptophan.*

Flask.	4-MT 2500 × 10^{-6} M.	Tryptophan × 10^{-6} M.	Growth in hours or days. 18	23	44	3	4	6	R. T/4-MT.
1	0	0	0	0	0	0	0	0	—
2	0	39	+++	+++	+++	+++	+++	+++	—
3	0	20	+++	+++	+++	+++	+++	+++	—
4	0	10	++	+++	+++	+++	+++	+++	—
5	+	625	+++	+++	+++	+++	+++	+++	1/4
6	+	312	++	+++	+++	+++	+++	+++	1/8
7	+	156	tr	+++	+++	+++	+++	+++	1/16
8	+	78	0	0	++	+++	+++	+++	1/32
9	+	39	0	0	0	0	0	+++	1/64
10	+	20	0	0	0	0	0	0	1/128

EXP. 8.—*Tryptophan/4-MT Relations; Titration of 4-MT.*

Tryptophan constant at 52 × 10^{-6} M. This experiment was carried out simultaneously with Exp. 5.

Flask.	4-MT × 10^{-6} M.	Growth in hours or days. 22	27	46	4	5	R. T/4-MT.
1	0	++	+++	+++	+++	+++	—
2	6667	0	0	0	0	0	1/128
3	3333	0	0	0	0	0	1/64
4	1667	0	0	0	++	++	1/32
5	833	0	0	+++	+++	+++	1/16
6	416	+	++	+++	+++	+++	1/8
7	208	++	+++	+++	+++	+++	1/4

However, it is at least probable that 4-MT has no effect on the condensation of indole and serine in the synthesis of tryptophan, but that its action is due to competition with tryptophan in some further essential reaction.

If these experiments were related to isolated enzymes it would be expected that the concentration of indole and serine should affect the concentration of tryptophan synthesized, and so show the same competitive relations with 4-MT as does tryptophan. In growth experiments, however, the concentration of tryptophan synthesized is probably a constant character of the cell, and does not vary with the concentration of metabolites present so long as these are adequate to produce a "normal" result. A bacterial inhibitor can therefore be expected to have quantitative relationships only with its immediate competitor, and not with precursors whose normal metabolism may be dependent upon a number of other factors.

THE SIGNIFICANCE OF THE POSITION OF THE METHYL GROUP IN METHYL-INDOLE AND METHYL-TRYPTOPHAN.

As indicated in Exp. 1, the inhibitory potencies of 4-, 5-, 6-, 7-MI are in the order 4>6>7>5. This is judged by the time lag in growth. It is confirmed by taking opacity readings in the early stages (21 hours) even with excess of indole (I/MI ratio 2/1).

	Bacteria/c.c. $\times 10^8$.*
Control without MI . . .	8·7
+ 4-MI . . .	4·0
+ 6-MI . . .	5·1
+ 7-MI . . .	6·6
+ 5-MI . . .	7·5

* Converted from Hilger "Spekker" absorptiometer readings.

1-, 2- or 3-MI had no demonstrable power of inhibition. On the other hand, as indicated in Exp. 2, 2-MT had no inhibitory action, while 4-, 5-, 6-, 7-MT all inhibited in the order 4>5>6>7.

The present differs from most work on the inhibitory action of metabolite analogues in the completeness of the ranges of analogues studied, and is thus particularly well suited for deducing the details of the mode of action of these inhibitors. It has been shown that the methyl-indoles act by interfering with the further utilization of indole (by reaction with serine to form tryptophan), and the methyl-tryptophans by interfering with the further utilization of tryptophan. In both series, differences in the inhibitory powers of isomers must be due to differences in their reactivity towards the enzymes concerned in the processes inhibited; the greater the reactivity of the metabolite analogue towards the enzyme the greater will be its inhibitory activity.

It will be convenient to discuss the methyl-indoles first. These fall into two groups, viz. those (1-, 2- and 3-methyl-indoles) with methyl groups attached to the pyrrole ring and those (4-, 5-, 6- and 7-methyl-indoles) in which the methyl groups are attached to the benzene ring. The first group is entirely non-inhibitory, a finding which suggests that the combination of indole with the enzyme very closely involves the pyrrole ring, any substitution in which causes, perhaps for stereochemical reasons, a complete failure of the modified substrate to combine with the enzyme. All the members of the second group are inhibitory, although to varying degrees, and it seems that substitution in the benzene ring does not prevent combination of the substituted indole with the enzyme, although

it does profoundly affect its reactivity towards the enzyme. The alternating effect observed in the order of the inhibitory activities of the members of this second group (4-Me>6-Me>7-Me>5-Me) at once suggests that the underlying mechanism is polar in character.

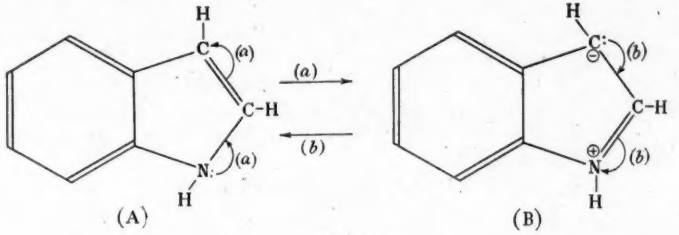


FIG. 2.

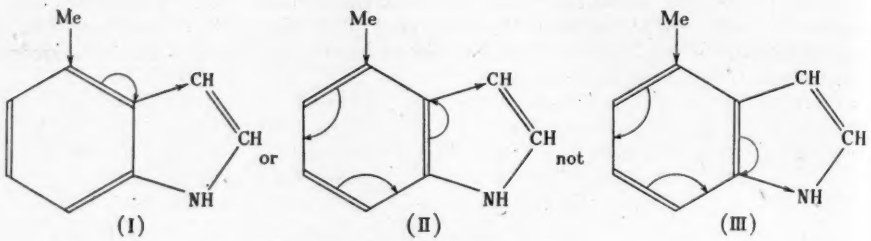
According to present views the indole molecule is to be regarded as having a structure intermediate between the two forms (A) and (B) (Fig. 2), which may, formally only, be interconverted by the electron shifts (*a*) and (*b*)*.

The proportion in which these two extreme forms contribute to the true state (" resonance hybrid ") of the indole molecule will influence its chemical behaviour, including its reactivity towards enzymes, and will depend on the nature and position of any substituents. Substituents tending to induce electron shifts favouring (*a*) and opposing (*b*) result in an increased proportion of (B) in the substituted indole as compared with indole itself, while substituents favouring (*b*) conversely result in an increased proportion of (A).

The methyl group is known to repel electrons, a tendency which may be represented by the symbol Me⟶ (for simplicity this notation is used here, in the case of the methyl group, to include both inductive and tautomeric effects). In consequence, a methyl group attached to a benzene ring will initiate, or facilitate, a series of electron displacements calculated to relieve this tendency. For this reason the first displacement will be Me ⟶ C(=C↑)C , not Me⟶ C(=C)C

Further, similar displacements will occur in the same sense, subject always to

* It may not be out of place to indicate briefly the meaning of the various symbols employed in the electronic argument. Straight arrows, as A ⟶ B, indicate a movement of electrons away from A and towards B while remaining within the electron octets of the atoms A and B ; thus A : B passes into A : B. Curved arrows indicate a tendency for a pair of electrons to migrate from the octet of one atom to that of another ; thus A ⟵ B — C denotes a change from A ⋮ B : C to A : B ⋮ C, and A ⟵ B a change from A ⋮ B to A : B̈. Straight arrows are used for what are known as " inductive " effects and curved arrows for " tautomeric " effects ; in the case of the methyl group the inductive effect involves the change from $H_3C : C$ to $H_3C : C$ and the tautomeric effect that from $H : \ddot{C} : C$ to $H \ddot{C} \vdots C$, i.e. from $CH_2 — C$ to $H^+ CH_2 = C$. In all cases it must be borne in mind that, although the transferences are not complete, they must be such that the completed process would not result in any carbon or nitrogen atom becoming devoid of at least a share in an octet of eight valency electrons ; this requirement is the octet rule.



(I) (II) (III)

FIG. 3.

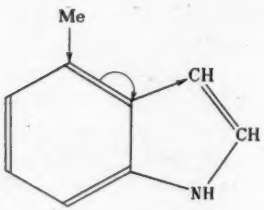
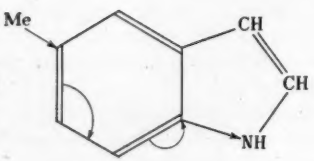
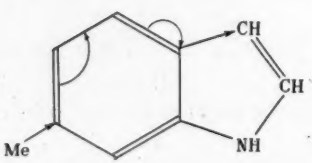
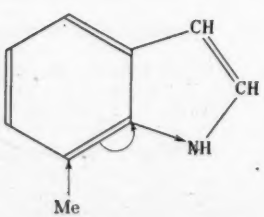
		Effect on process (*a*)	(*b*)	Effect on proportions of (A) and (B) in hybrid.
4-Me		Opposed	Favoured	More (A)
5-Me		Favoured	Opposed	More (B)
6-Me		Opposed	Favoured	More (A)
7-Me		Favoured	Opposed	More (B)

FIG. 4.

the proviso that the carbon atoms must retain their electron octets. In the case of 4-methyl-indole we thus have (I) or (II) (Fig. 3), both of which, it will be seen, lead to the same (clockwise) resultant direction of flow of electrons in the pyrrole ring.

The other alternative (III) is excluded by the octet rule. It is clear, and can be confirmed by the reader for the other methyl-indoles, that the final result is not affected by the manner in which the bonds in the benzene ring are written (as (I) or as (II)). The effect of these tendencies on the electronic shifts (*a*) and (*b*) (Fig. 2), and consequently on the proportions of (A) and (B) in the resonance hybrids, is indicated for the four methyl-indoles under consideration in Fig. 4, indole itself being taken as the standard.

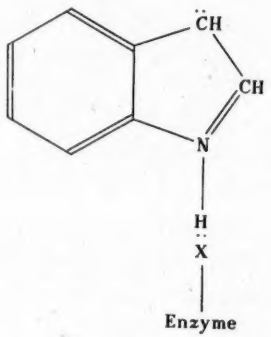


FIG. 5.

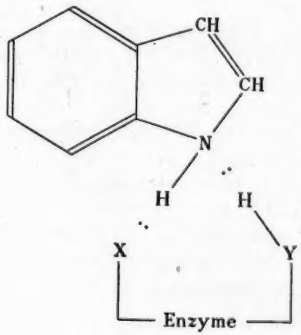


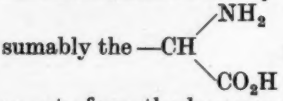
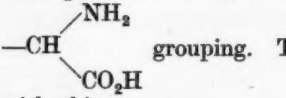
FIG. 6.

If we compare these conclusions with the experimental findings it is seen that the two most inhibitory compounds (4-methyl and 6-methyl-indoles) are those in which (A) preponderates; the results thus show that the nature of the combination of indole and the methyl-indoles with the enzyme is such that it is favoured by structure (A) rather then structure (B).

Now it is highly probable (Pauling, 1940) that enzyme-substrate combinations involve, not ordinary co-valencies, but hydrogen bonds. In the indole molecule only the = NH group is at all likely to enter into hydrogen bonds, and there is a very real difference between (A) and (B) in their power to form such bonds. For, whereas (B) can only form a single bond of this type (Fig. 5), (A) can enter simultaneously into two hydrogen bonds (Fig. 6).

The results of the present work with the methyl-indoles seem to indicate, therefore, that the combination of indole with the enzyme concerned is of the latter type, involving attachment by two hydrogen bonds to two side-chains of the enzyme molecule (calculation shows that there is no stereochemical difficulty); such double attachment, besides being more stable than attachment by a single hydrogen bond, would result in a more rigid anchoring of the indole molecule into position for further reaction; it would not be surprising if two-point attachment in this, or in some similar manner, proved to be a general feature of enzyme-substrate combinations.

Turning now to the methyl-tryptophans the results are very different, the

order of inhibitory power being 4-Me>5-Me>6-Me>7-Me; it seems, therefore, that attachment is not by double hydrogen bonding on the = NH group, and presumably the  grouping of the side-chain is concerned. Since this is remote from the benzene ring, to which it is not connected by a system of conjugated double bonds, any effect of polar substituents in the benzene ring would be expected to fall off simply with the distance of the substituent from the  grouping. The order observed experimentally is quite consistent with this.

Finally, brief mention may be made of some preliminary observations of our colleague, Dr. D. Herbert, on the action of *Bact. coli* tryptophanase on the methyltryptophans; the order of efficacy of these substances as substrates for this enzyme system was 4-Me>6-Me>5-Me>2-Me>7-Me. The alternating order observed for the compounds methylated in the benzene ring suggests that the combination with this degradative enzyme, unlike that with the synthetic enzyme of *Bact. typhosum*, involves double hydrogen-bonding on the = NH group, as in the case of the methyl-indoles.

The considerations developed above are capable of obvious extensions to substituents of differing polar character and to other inhibitory metabolite analogues; these extensions are in process of exploration.

CONCLUSIONS.

1. Introduction of a single methyl group into the benzene rings of indole and tryptophan converts these essential metabolites into bacterial inhibitors.
2. The methyl-indoles obstruct competitively the further use of indole in the synthesis of tryptophan. The methyl-tryptophans obstruct the further use of tryptophan in protein synthesis.
3. The degree of potency depends upon the position of the substitution.
4. The bearing of the findings upon the theory of fixation of substrates by enzymes is discussed.

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PROTECTIVE ANTIGENS ISOLATED FROM *BR. ABORTUS.*

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MILES and Pirie (1939*a*) described a soluble antigen prepared from *Brucella melitensis*. In its most highly aggregated form it appeared to be a complex of at least two phospholipides with a protein-like material and the N-formyl derivative of a polyhydroxyamine. Mention was made (Miles and Pirie, 1939*a*, *b*) of a similar material obtained from *Br. abortus*, but the behaviour of this antigen was at that time irregular and no details of its preparation and properties were given. The differences that were observed in the biological behaviour of the melitensis antigen (Miles and Pirie, 1939*c*) in different states of aggregation, taken in conjunction with the general body of immunological experience, suggested that the relative inefficiency, as immunizing agents, of preparations made from *Br. abortus* by other workers, might be due to the fact that the antigens in them were not in the optimum state of aggregation. Working on this hypothesis many preparations of the abortus antigen have been made during the past seven years by different methods, and in this paper techniques are described whereby antigens capable of conferring a considerable degree of immunity to guinea-pigs can be prepared.

For the isolation of the antigen it is unnecessary completely to disintegrate the organisms, and for this and other reasons Miles and Pirie (1939*c*) suggested that in *Br. melitensis* it might exist as a capsule. The same arrangement of the antigen is probable in abortus organisms, and Huddleson (1940, 1941) has now presented histological evidence for a capsule.

One strain of *Br. abortus* organisms has been used throughout this work. In the earlier work on *Br. melitensis* (Miles and Pirie, 1939*a*, *b*, *c*) only one strain was used. The differences in chemical and physical properties to be described may, therefore, be strain and not species differences.

Preparation of the Antigen.

Suspensions were prepared in neutral 0·5 per cent phenol saline from 48–72 hours' liver agar growths of strain 544 (McEwen, Priestley and Paterson, 1939). This strain is virulent, smooth, and requires 5–10 per cent CO_2 for good growth. The cultures used as seed material were the second subcultures from a dried culture (the fourth subculture of the original isolation on 26/3/36), except where an early variant, able to grow without additional CO_2, was chosen for comparative purposes.

Part of the antigen is extracted by the 0·5 per cent phenol, but this extract contains the greater part of the agar breakdown products and other materials that complicate the purification of the antigen. Unless economy of material is

essential, therefore, it is better to centrifuge the suspension and discard the fluid, or keep it for the preparation of largely disaggregated antigen by the methods described later. The bacterial mass left after centrifuging is suspended in 20 times its weight of neutral 2 per cent phenol, and the mixture is left, with occasional shaking, for 1–2 weeks. After centrifuging for an hour at 3500 r.p.m. the supernatant fluid is only slightly opalescent and contains most of the readily extractable antigen; further similar extraction with 2 per cent phenol brings out little more. There is no evidence that the use of stronger phenol increases the yield.

The antigen can be isolated by the direct ultracentrifugation of this extract, but this involves centrifuging inconveniently large volumes of fluid; it is, therefore, better to concentrate the extract first by ultrafiltration. "Cellophane" sausage skin tube 2·2 in. in circumference is suitable if it is protected from hydrostatic stretching by being surrounded by a cloth tube of the same diameter. The tube is knotted at one end, the other end tied firmly on to a rubber bung carrying a filling tube, and the whole is mounted in a bottle that can be exhausted and left clipped off. If the tube is kept filled and if its inside is at atmospheric pressure while its outside is at about 20 mm. pressure, the initial rate of ultrafiltration is about 1 ml. per hour per cm. length of tube. Slight clogging during filtration reduces the rate of flow, but this can be partly restored by releasing the vacuum and, with the tube full of fluid but flaccid, rubbing the two internal faces together gently. The fluid is ready for ultracentrifugation when there is a significant rise in its viscosity; at this stage it will have a solid content of 1 to 2 per cent. The pH is adjusted to 7, and it is centrifuged for 30 min. at 10,000 r.p.m. to get partial clearing.

An air driven ultracentrifuge of the type described by Masket (1941) was used to sediment the antigen; in general the centrifuge runs lasted for 30 min. at 30,000 r.p.m. (R.C.F. 50,000). The supernatant fluid is poured off from the turbid coherent pellets, and these are resuspended in water to give a fluid with a solid content of less than 1 per cent and ultracentrifuged again. On a third similar ultracentrifugation there is only a trace of unsedimented material in the supernatant fluid; no further fractionation is therefore possible by ultracentrifuging under these conditions. The pellets are resuspended in water, and cleared by centrifuging for 30 min. at 10,000 r.p,m. It is the resulting colourless opalescent fluid that is referred to as the antigen; solutions are stable at 0° C. for some weeks, but after some months part of the material flocculates. Bacteria do not grow in it readily, and it can be satisfactorily preserved with 0·5 per cent phenol.

Digestion with commercial trypsin or autoclaving liberates further quantities of antigen from the bacterial mass left after extraction with 2 per cent phenol. After 3–4 days' digestion at pH 8 and 40° C. in the presence of chloroform as disinfectant the bacterial suspension is centrifuged and the antigen isolated by differential centrifugation as before. The residue from this treatment is resuspended and is autoclaved for 15 min. at 120° C. at an initial pH of 8·5. The pH is of some importance, for it falls during autoclaving and the antigen is destroyed below pH 6·5. If the suspension is made in 0·1 M phosphate buffer at 8·5 the final pH will not be below 7·5. This extract also is worked up as before. These two products should be kept separate from that made from the 2 per cent phenol extract, for one of them may be contaminated with constituents of the commercial trypsin, and the other is certainly contaminated with antigenically inert fragments of the bacterium. The bacterial bodies are partly disintegrated by

autoclaving, and if the fluid obtained by autoclaving a second time is fractionated ultracentrifugally, a product with the physical properties associated with the antigen can be separated. Its chemical properties are, however, clearly distinguishable from those of the antigen.

Batches vary somewhat both in the total yield of antigen and in the way in which it is distributed between the different extracts. Good yields, expressed as a percentage of the dry matter of the bacterial sediment in the original 0·5 per cent phenol suspension are :

1	per cent	from the	0·5 per cent phenol extract.
7	,,	,,	2 per cent phenol extract.
2·5	,,	,,	trypsin extract.
2·5	,,	,,	extract on autoclaving.

Under the best conditions the yield of antigen may thus be 13 per cent of the dry weight of the killed bacterium, and this yield is of the same order as that found with *Br. melitensis*. Yields may, however, fall to half of these values.

CHEMICAL AND PHYSICAL PROPERTIES OF THE ANTIGEN.

In its purified form the antigen is soluble over the whole pH range, but it readily precipitates from crude solutions along with any other material that is acid precipitable. Unlike solutions of the *Br. melitensis* antigen this material does not show anisotropy of flow. Solutions are apparently unaffected by heating for periods up to an hour at 100° C. in the neutral range, and 60 per cent of a preparation can be recovered by ultracentrifuging a solution that has been autoclaved for an hour at 120° C. and pH 7.

Preparations made by the methods described are ultracentrifugally inhomogeneous, as would be expected if they consist of particles derived by the more or less random fragmentation of a bacterial capsule. By centrifugation at an intermediate speed, such as 20,000 r.p.m., some fractionation is possible. The fractions do not differ from one another fundamentally in their chemical properties ; their biological properties have not been compared.

After drying or freezing part of the antigen becomes insoluble, and all products that are to be used as immunizing agents or for serological studies have therefore been kept in solution. The antigen, unlike most other large molecules, cannot be satisfactorily dried by sublimation, for it forms a loose incoherent mass that is scattered by the wind from the evaporating ice. Analytical and biological data depend on dry weights measured by evaporating samples of the salt-free solution to dryness at room temperature over P_2O_5 *in vacuo*. Conditions were such that the dried film was less than 0·1 mm. thick ; drying is likely to have been complete throughout, and frequent checks showed no further loss of weight on drying at 100° C. These films are hygroscopic, but do not pick up water with the avidity of the more open textured mass that results from drying by sublimation. About 5 per cent of water is absorbed by the time weighing is finished ; the results have not been corrected for this moisture content.

The nitrogen content is 4–5 per cent, but, as with other materials of this type, it is not a trustworthy index of contamination, for the probable contaminants

have similar nitrogen contents. Carbohydrate, estimated by an orcin method (Pirie, 1936), using glucose as standard, falls in the range 10–12 per cent, but the high lipid content makes this method somewhat uncertain on account of the slight turbidity of the final coloured solution. The main value of the determination is to detect contamination with agar; this gives a cherry red colour in the early stages of the estimation, and any preparation which gives this colour or contains more than 13 per cent of carbohydrate can probably be further purified by ultracentrifugation. The formyl content is a trustworthy index of the presence of antigen, for formic acid is not known to be liberated, under the conditions used, from any other substance except the melitensis antigen. 10–20 mg. lots of antigen are hydrolysed for 30 min. at 100° C. with 1–2 ml. of N H_2SO_4, and formic acid is estimated (Pirie, 1946) on an aliquot of the hydrolysate. Agar yields formic acid slowly when heated with acids, but the amounts that can remain in a purified preparation of antigen introduce a negligible error if the hydrolysis only lasts for 30 min. Values range from 4 to 5 per cent of CHO — Lower values are given by products made by autoclaving the bacterial residue because these are contaminated with fragments of the bacterium; higher values will be given by products from which the loosely bound phospholipid has been partially removed.

The phosphorus content varies in the range 0·7 to 2·0 per cent. Most of the phosphorus is present as phospholipid, and it can be separated from the antigen by treating an aqueous solution with 10 volumes of a mixture of alcohol and ether containing 0·5 per cent of concentrated hydrochloric acid (Miles and Pirie, 1939*a*). The defatted residue is fairly constant in its properties. It is now only partially soluble in water, and contains 0·5 per cent of phosphorus. Half of this is liberated as phosphate after hydrolysis for an hour at 100° C. in N/12 HCl; the other half remains soluble after this treatment, but is present in the amorphous precipitate that separates on hydrolysis with stronger acid. This precipitate closely resembles the one prepared in the same way from the melitensis antigen and called PL2 (Miles and Pirie, 1939*c*).

The variability of the phosphorus content of the antigen is due to variations in the amount of phospholipid associated with it and in an extensive series of analyses products with satisfactory centrifugal behaviour and formyl content have always contained about 0·5 per cent of phosphorus not soluble in lipid solvents. Products made from the 2 per cent phenol extract have a higher phospholipid content than those made by the other three methods of extraction, and the more rapidly sedimenting fraction of a preparation generally has a higher phosphorus content than the more slowly sedimenting part. No sharp segregation into fractions of different phospholipid content has, however, been possible. Some of the nitrogen in the antigen occurs in these two phospholipid fractions, but the greater part remains soluble after acid hydrolysis, and this solution gives the Elson and Morgan (1933) colour reaction for amino-sugars. As with the corresponding product from *Br. melitensis* (Miles and Pirie, 1939*d*), the colour reaction is given without preliminary acetylation; there is, therefore, reason to think that the substance present is not one of the already known amino-sugars.

In the course of preparation the antigen is only subjected to ultracentrifugal fractionation, and this is designed to give a group of particles having sedimentation constants within a fairly broad range. Other methods of fractionation which might be of use both in the preparation and as criteria of homogeneity have

therefore been sought, but so far without success. Precipitation with alcohol or acetone removes part of the lipid and makes much of the antigen insoluble in water. Precipitation with ammonium sulphate at 40 per cent of saturation does not appear to alter the antigen if the precipitate is centrifuged off and redissolved in water in a few minutes, but if contact with ammonium sulphate of this concentration lasts for some hours much of the antigen is so altered as to be no longer soluble in water. Solubility can be restored by washing the precipitate free from ammonium sulphate and heating it in neutral aqueous suspension at 100° C. for some hours. Preparations that have been disaggregated by autoclaving or treatment with organic solvents can also be partially reaggregated by exposure to half saturated ammonium sulphate solution.

This behaviour with ammonium sulphate complicates the separation of the small amount of antigen from the original 0·5 per cent phenol extract of the bacteria. In a few experiments part of this extract has been directly ultracentrifuged, but the volume is too large for this to be a satisfactory routine procedure. Agar breakdown products in it precipitate with ammonium sulphate, but this precipitate does not redissolve unless the ammonium sulphate concentration is lowered below 1/10th of saturation. To separate the antigen, therefore, 360 g. of ammonium sulphate is added to each litre of extract; the precipitate is centrifuged off immediately and suspended in 4–5 times its bulk of water. After some hours it is centrifuged; the fluid contains much of the antigen, and this can be recovered by dialysis and ultracentrifugation. Alternatively the ammonium sulphate precipitate can be mixed with phenol or pyridine to disaggregate and dissolve the antigen; from this solution it is precipitated by alcohol, and can be purified by ultracentrifugation after partial aggregation with ammonium sulphate. By these procedures it is possible to recover only about half the antigen that can be separated by direct ultracentrifugation.

Disaggregation of the Antigen.

In some of the biological tests to be described later, disaggregated products have been used. A brief account of the methods used in their preparation is therefore necessary, but no conclusions about the nature of the differences between fractions with different physical properties have yet been reached.

Many disaggregating agents have been tried, but only concentrated phenol, pyridine and shaking with chloroform and amyl alcohol have been found to give reproducible results. In each case the treatment results in the separation of the antigen into two main fractions. The optically homogeneous solution that results when an aqueous solution of antigen is mixed with six parts of phenol or pyridine at room temperature gives a clear gelatinous layer when centrifuged at 3000 r.p.m. Disaggregated antigen can be recovered from this gelatinous layer by extraction with water, after removal of the reagent by washing with alcohol on the centrifuge. Similarly, part of the antigen, disaggregated, is present in the emulsion layer that is formed when the antigen, dissolved in acetate buffer at pH4, is shaken with chloroform and amyl alcohol (Sevag, 1934). The material soluble in phenol or pyridine can be precipitated by alcohol, and these precipitates dissolve freely in water. All the solutions thus obtained are clearer than those of the original antigen at the same concentration, and they are incompletely sedimented even when centrifuged at 40,000 r.p.m. A few comparisons have been made of the sedimentability of the original and disaggregated antigen at

24,000 r.p.m. Whereas 80–90 per cent of the former sediments into a coherent pellet in 30 min., only a third of the latter does so. There is no regular difference between products disaggregated by the three different methods, nor between the different fractions that result from each technique of disaggregation. In the biological tests reported only that part of a disaggregated preparation that sedimented at 24,000 r.p.m. was used.

Disaggregated antigen, from which the loosely bound phospholipid had been separated, was easily made from the *melitensis* antigen by treatment with 80 per cent acetic acid (Miles and Pirie, 1939*a*). This treatment is unsatisfactory with the abortus antigen because it is not soluble in strong acetic acid, and, although the treatment disaggregates part of the antigen, most of it is rendered insoluble in water. Disaggregated melitensis antigen was polydisperse, but it behaved towards precipitating agents like a single substance, and could be used as a convenient form of the antigen both in its isolation and in the study of many of its biological properties. It is clear from this survey that no such method of converting the abortus antigen into a water-soluble material of relatively low molecular weight has as yet been found.

BIOLOGICAL PROPERTIES.

Inoculation of the Antigen into Normal Animals.

(a) *Rabbits.*—Intradermal inoculation of 0·1 to 1 mg. produced small hard swellings at the site of inoculation at the 48th to 72nd hours. With certain preparations there was very slight necrosis of the superficial dermis at the 72nd hour, but all sites of injection had resolved by the 10th day without the skin breaking. In many animals, although not constantly with any given dose, a secondary oedematous inflammatory reaction was noted at the site of inoculation about the 9th–11th day. These swellings resolved in from 7–10 days, and presumably were due to circulating antibodies reacting with residual traces of antigen in the skin. Agglutinins were developed, reaching their maximum titre (1 : 640–1 : 1280) between the 6th and 11th days. By the 60th day they had dropped to 1 : 20–1 : 40. The inoculation of small single doses of the antigen by the intramuscular, intravenous or subcutaneous route provoked similar agglutinin responses.

The effect of repeated intradermal inoculation on the circulating agglutinins has been studied. In the first experiment groups of two rabbits were given a total dose of 0·6 mg. of the antigen as one, two and four doses at intervals of 28 days. Severe local reactions followed at the sites of the repeated inoculations. Necrotic areas up to 2 cm. in diameter developed, which subsequently sloughed. The titre continued to fall for four days after each repeat dose, and thereafter rose to 1 : 640 or 1 : 1280 during the course of a further 6–11 days, after which the titre again receded. Sixty days after the last portion of the total dose of 0·6 mg. had been inoculated the titres were at 1 : 20 to 1 : 40 level, at which height they remained for a further 60 days, when observation ceased.

In the second experiment a rabbit was given four doses of 0·3 mg. at intervals of 10–11 days. Three others were similarly inoculated with four doses of 0·03 mg., 0·003 mg. and 0·0003 mg. respectively. Local reactions were noted following the repetition of the 0·3 mg. doses, but not with those of smaller amount. The 0·3 mg. doses (repeated) at 10–11 day intervals maintained the titre at an almost constant level of 1 : 640–1 : 1280 from 7 days after the first dose until

10 days after the last. The rabbits inoculated with 0·03 mg. and 0·003 mg. developed titres similarly, but at a lower level, viz. 1 : 80–1 : 160. With 0·0003 mg. the titres failed to rise above 1 : 40.

In a third experiment three rabbits were given five doses of 0·3, 0·03 mg. and 0·003 mg. respectively at 7-day intervals, and the same treatment was applied to three rabbits at 3-day intervals. The effect of repeating these inoculations at short intervals was to increase agglutinin production slightly and to delay the maximum until about the 12th day. The rabbits inoculated with 0·3 mg. at 3- and 7-day intervals had maximum titres of 1 : 5000 on the 12th day after the first inoculation. The titres receded to 1 : 1280 by the 18th day. In the 3-day interval rabbit this recession was continuous but slow. The titre of the 7-day interval rabbit remained steady at 1 : 1280 from the 18th to the 44th day and then dropped slowly. At 85 days both rabbits had titres of 1 : 160. The agglutinin response in the rabbits inoculated with the smaller doses was in all respects similar to the above but on a lower level: with 0·03 mg. the maximum titre recorded was 1 : 640, and with 0·003 mg. 1 : 320 on the 10th and 12th days. Residual titres on the 89th day were 1 : 40–1 : 80.

The inoculation of larger amounts of antigen (3–10 mg.) caused transient inappetance and some loss of bodily condition for a few days. A large oedematous and erythematous swelling up to 5 cm. in diameter developed within 24 hours, and by the 72nd hour the centre of the swelling was necrotic (1 × 1 cm.) and umbilicated. About the 8th–10th day the necrotic centre sloughed away, releasing a variable quantity of pus. Healing took 4–6 weeks.

Repeated intramuscular inoculation of small doses did not cause any observable symptoms.

(b) *Guinea-pigs.*—Intradermal inoculation 0·1 to 1 mg. of antigen produced an evanescent local reddening. Inoculation of 3 to 10 mg. caused a more severe local reaction with necrosis, followed by rapid healing. In many animals secondary reactions were noted, as in rabbits.

(c) *Cattle.*—Two normal yearling calves which were inoculated intradermally with 1 mg. of antigen developed hard, non-painful swellings which disappeared very slowly, some trace of them still being discernible after 6 weeks. The two animals developed their maximum titre (1 : 640) within 6 days after inoculation. Sixty days after inoculation their titres were 1 : 40.

Inoculation into Sensitized Animals.

Small doses of antigenic fractions of varying molecular size have been inoculated into rabbits and calves which had been sensitized 28 days earlier by subcutaneous inoculation, with suitable doses of living *Br. abortus*, for which purpose a smooth aerobic strain of moderate virulence (McEwen, 161) was used. The reactions of these sensitized animals were much more vigorous than those of normal animals.

In rabbits local oedema and reddening was observed at the 24th hour, and by the 72nd hour this had progressed so that a necrotic lesion approximately 1 cm. across was seen. This was followed in 4–7 days by scab formation. Subsequent healing was slow, taking 20–30 days, but there was no pus formation.

A series of nine sensitized and six normal control calves was inoculated intradermally into the skin of the neck region. The technique used in these experiments was essentially the same as that employed in carrying out the intradermal

test with tuberculin, the increase in the thickness of a fold of skin at the point of inoculation being determined by calipers. In the sensitized calves soft swellings developed, which were oedematous and painful, but they did not become necrotic. They reached their maxima at the 72nd hour, the skin thickness being then increased up to 12 mm. with the most active preparations. Resolution was slow, the skin not returning to normal for a period of 24–48 days. The swellings which developed in the control animals were hard and circumscribed and reached their maxima in 48 hours, fading greatly by the 72nd hour.

Immunization Experiments in Guinea-pigs.

Preliminary small scale experiments, that need not be reported in detail, showed that fractions made from the bacteria by many different procedures did, in varying degree, confer protection on guinea-pigs against subsequent parenteral infection with virulent organisms. These experiments also suggested that the strain, age, sex and weight of the guinea-pig affected the protection. In the experiments reported animals of the same strain and comparable age and weighing 300–400 g. were used. At the chosen interval after treatment with the substance under investigation, the group of animals was divided into three subgroups. Except in the experiment recorded in Table I these received, as test dose intramuscularly in the thigh, 1 ml. of 1 in 10^{-5}, 1 in 10^{-6}, 1 in 10^{-7} dilutions respectively of a saline suspension of a 48–72-hour growth of strain 544 standardized by opacity to tube 3 on Brown's scale. In the experiment shown in Table I the doses were 1 ml. of 1 in 10^{-6}, 1 in 10^{-7} and 1 in 10^{-8} dilutions. Immediately before injecting the test doses, viable counts were made on blood agar plates or by inoculating "roll" tubes made with liver agar. These counts showed that the test doses contained approximately 40,000, 4000, 400 and 40 organisms.

TABLE I.

Product.	Dose.	Infection rate.		
10·3 mg. of washed phenol killed bacteria .	10^{-6}	1/10	10%	
	10^{-7}	5/10	50%	9/30 30%
	10^{-8}	3/10	30%	
0·3 mg. of a 2 per cent phenol extract precipitated by ammonium sulphate and fractionated by ultracentrifugation	10^{-6}	1/10	10%	
	10^{-7}	3/10	30%	4/30 13%
	10^{-8}	0/10	..	
0·3 mg. of a 2 per cent phenol extract fractionated by ultracentrifugation	10^{-6}	2/9	22·2%	
	10^{-7}	7/10	70%	10/28 35%
	10^{-8}	1/9	11·1%	
Controls . . .	10^{-6}	10/10	100%	
	10^{-7}	10/10	100%	29/30 96·6%
	10^{-8}	9/10	90%	

Notes.—(1) In the above and in subsequent tables the denominator indicates the number of animals exposed to infection and the numerator the number infected.

(2) The test doses are expressed as dilutions of a suspension standardized by opacity to correspond with tube 3 on Brown's scale. The volume of the infecting suspensions employed was 1 c.c., the 10^{-6} dilution containing approximately 4000 viable organisms.

Six weeks after infection all the animals were killed, the blood agglutinin titres determined, and the spleens ground up in 1 per cent broth-saline and cultured for *Br. abortus*. The presence of live organisms in the spleen has been

accepted as evidence that any immunity engendered had been overcome. The immunizing values of the various fractions or of different methods of immunization have been assessed by a comparison of the infection rates.

In Table I two products are compared with a quantity of killed organisms containing as much or more antigen ; it is clear that the partially purified antigen is as effective as the killed organisms. Two products, derived from the same

TABLE II.

Product.	Test dose.*	Interval between test dose and killing. 6 weeks.	9 weeks.	12 weeks.	Total infection rate.
Aggregated, 3 mg. i.d.	10^{-5}	2/4	0/1		
	10^{-6}	2/4	1/4	0/1	6/22 27%
	10^{-7}	0/4	1/4		
Disaggregated, 1 mg. i.d.	10^{-5}	3/4	2/2		
	10^{-6}	3/4	3/4	1/1	16/20 80%
	10^{-7}	3/4	1/1		
Controls	10^{-5}	6/6	6/6	6/6	
	10^{-6}	6/6	6/6	6/6	53/54 98%
	10^{-7}	5/6	6/6	6/6	

* The test dose was administered thirteen weeks after the vaccine dose.

TABLE III.

Product.	Dose in mg. dry matter.	Test dose.	Cambridge.	Weybridge.	Total infection rate.
2 per cent phenol extract precipitated with ammonium sulphate and redissolved by heating	0·3 i.d.	10^{-5}	8/9	8/12	16/21 76%
		10^{-6}	8/10	5/14	13/24 54% 33/68 48%
		10^{-7}	3/10	1/13	4/23 17%
Extract from bacterial residue by trypsin digestion and autoclaving	0·3 i.d.	10^{-5}	7/10	7/12	14/22 64%
		10^{-6}	5/10	2/14	7/24 29% 22/67 33%
		10^{-7}	0/8	1/13	1/21 5%
Controls	..	10^{-5}	10/10	12/12	22/22 100%
	..	10^{-6}	10/10	12/12	22/22 100% 65/66 98%
	..	10^{-7}	9/10	12/12	21/22 95%
Number of viable brucella in 1 c.c. of 10^{-6} dose			3700	4360	

Note.—The products were prepared from strain 544A (aerobic).

batch of organisms, and which were fractionated by differential centrifugation so as to yield an aggregated and a disaggregated fraction were compared and the results are set out in Table II. It is clear that the former is the more effective. The efficacy of antigens prepared from the 2 per cent phenol extract of the bacteria and from the bacterial residue after this extraction by digestion with trypsin and autoclaving is shown in Table III to be comparable. These two products, unlike all the others for which the results of tests are given here, were made from a variant of strain 544 that did not need CO_2.

A product prepared by digestion with trypsin and autoclaving a suspension of a CO_2 culture of 544 was selected for an investigation into the effect of varying the time interval between a single dose of the antigen and infection; of giving two doses of antigen and of giving a single larger dose.

TABLE IV.

Treatment.	Test dose.	Cambridge.	Weybridge.	Total infection rate.	
A					
Single dose 8 weeks before infection. 0·3 mg. in 0·2 c.c.	10^{-5}	8/10	4/10	12/20 60%	21/59 36%
	10^{-6}	6/10	3/10	9/20 45%	
	10^{-7}	0/9	0/10	0/19 0%	
B					
Single dose 4 weeks before infection. 0·3 mg. in 0·2 c.c.	10^{-5}	7/10	5/10	12/20 60%	18/60 30%
	10^{-6}	3/10	2/10	5/20 25%	
	10^{-7}	1/10	1/10	1/20 5%	
C					
Single dose 2 weeks before infection. 0·3 mg. in 0·2 c.c.	10^{-5}	9/10	7/10	16/20 80%	27/60 45%
	10^{-6}	6/10	2/10	8/20 40%	
	10^{-7}	2/10	1/10	3/20 15%	
D					
Two doses 6 and 2 weeks before infection, each of 0·3 mg. in 0·2 c.c.	10^{-5}	4/10	3/10	7/20 35%	10/60 17%
	10^{-6}	1/10	0/10	1/20 5%	
	10^{-7}	1/10	1/10	2/20 10%	
E					
Single dose 2 weeks before infection. 0·6 mg in 0·4 c.c.	10^{-5}	7/8	9/10	16/18 89%	23/52 44%
	10^{-6}	2/7	4/10	6/17 35%	
	10^{-7}	0/7	1/10	1/17 6%	
Controls	10^{-5}	10/10	10/10	20/20 100%	60/60 100%
	10^{-6}	10/10	10/10	20/20 100%	
	10^{-7}	10/10	10/10	20/20 100%	
Viable count of 1 ml. of 10^{-6} dose		4700	3970		

Note.—The product used in this experiment was from CO_2-sensitive strain 544, and was made by incubating washed organisms with trypsin and autoclaving the residue.

The results of this experiment (Table IV) clearly indicated that the injection of two doses spaced by a month was superior to any of the single injection methods, and that probably the best time for a single injection was about four weeks before injection of the test dose. There was no indication that doubling the dose had any beneficial effect when given 14 days before infection. In a preliminary experiment we found that increasing the dose five times (to 1·5 mg. i.d.) did not give increased protection when the interval between vaccination and infection was 28 days.

There was, however, one feature of this experiment which calls for mention.

Earlier in this paper attention has been drawn to the severe reactions following the intradermal inoculation of the antigen into sensitized rabbits and guinea-pigs. One of the effects of the initial dose of 0·3 mg. in group D was to render a proportion of the guinea-pigs sensitive, and the inoculation of the second dose was followed by a marked local reaction. Twenty-four hours after inoculation the site was hot, red and painful, and about 2 cm. in diameter. By the 72nd hour it had enlarged to between 1·5 and 2·0 cm. The raised centre of the swelling was yellow, becoming necrotic. By the fifth day the centre of the swelling was purple, necrotic and definitely umbilicated. This necrotic tissue sloughed out. Healing was fairly rapid, usually being complete by the 15th day.

A further experiment was carried out in which the antigen was a mixture of aggregated products from several different lots of bacteria. Treatments B and D of Table IV were repeated. In order to find out whether the local reaction which follows a second injection could be avoided without reducing its effectiveness, by using the intramuscular route, groups were set up in which the primary doses were intradermal and subsequent doses intramuscular. It was also decided to observe the effect of the larger dose when given at what we believed to be the optimum time before infection, viz. one month. The effect of dividing this larger dose into four equal parts was also studied.

The intramuscular injections were performed in one of the hind limbs, the other being reserved for the inoculation of the test dose. The interval between infection and killing was approximately 42 days.

The local reactions to the second inoculation in Group B were severe, but in Groups E and F the local reactions to the second or subsequent inoculations were less marked. Rarely was necrosis and sloughing noted, but soft, raised, reddened swellings were always produced.

No apparent ill-effects followed the intramuscular inoculations.

The results, which are set out in Table V, showed again that there was an advantage in dividing the immunizing dose into two injections at an interval of 28 days, the last two weeks before test inoculation, but that the division of the immunizing dose into four parts had no particular advantage over two. The use of the intramuscular route for doses other than the first did not reduce the level of protection. The inoculation of 0·6 mg. had no advantage over 0·3 mg. when given as a single dose 28 days before infection.

DISCUSSION.

The studies of Miles and Pirie (1939*a*, *b*, *c*) and the work presented here show that *Br. melitensis* and *Br. abortus* contain antigens of a broadly similar nature which can be separated from the bacterial cell in relatively pure states. They have well defined physical and chemical characteristics, which can be used as criteria in estimating their probable value as immunizing agents.

Brucella extracts have been used by various workers. A trichloracetic acid extract prepared by Boivin's method from *Br. melitensis* was used by Roman (1938), who claimed some immunizing properties. The antigen was said to be non-protein in character and was called a glucolipid antigen. Priestley (1940) and Stahl and Hamann (1941), however, were unable to protect guinea-pigs with trichloracetic extracts of *Br. abortus*. Priestley's extract was antigenic, heat-stable and slightly toxic, but it failed to elicit skin reactions in infected guinea-pigs. He was also unable to obtain an antigen in this way from rough strains.

TABLE V.

Treatment.	Test dose.	Cambridge.	Weybridge.	Total infection rate.	
A					
Single dose 0·3 mg. i.d. 4 weeks before infection	10^{-5}	8/8	9/10	17/18 94%	30/56 54%
	10^{-6}	4/10	5/10	9/20 45%	
	10^{-7}	0/8	4/10	4/18 22%	
B					
Two doses 0·3 mg. i.d. 6 and 2 weeks before infection	10^{-5}	7/9	7/9	14/18 78%	24/55 44%
	10^{-6}	8/10	2/10	10/20 50%	
	10^{-7}	0/7	0/10	0/17 0%	
C					
One dose 0·6 mg. i.d. 4 weeks before infection	10^{-5}	7/7	9/10	16/17 94%	34/57 60%
	10^{-6}	7/10	5/10	12/20 60%	
	10^{-7}	4/10	2/10	6/20 30%	
D					
0·3 mg. i.d. 6 weeks before and 0·3 mg. i.m. 2 weeks before infection	10^{-5}	8/9	1/10	9/19 47%	20/59 34%
	10^{-6}	6/10	1/10	7/20 35%	
	10^{-7}	3/10	1/10	4/20 20%	
E					
4 doses each of 0·15 mg. i.d. given 14, 10, 6 and 2 weeks before infection	10^{-5}	7/9	5/10	12/19 63%	21/57 37%
	10^{-6}	6/10	0/10	6/20 30%	
	10^{-7}	2/9	1/9	3/18 17%	
F					
2 doses 0·15 mg. i.d. 14 and 10 weeks before infection followed by 2 doses 0·15 mg. i.m. 6 and 2 weeks before infection	10^{-5}	6/9	4/10	10/19 53%	21/56 38%
	10^{-6}	5/9	4/10	9/19 47%	
	10^{-7}	0/9	2/9	2/18 11%	
Controls	10^{-5}	7/7	9/10	16/17 94%	52/56 93%
	10^{-6}	9/9	10/10	19/19 100%	
	10^{-7}	9/10	8/10	17/20 85%	
Viable count of 1 ml. 10^{-6} dose		4120	5300		

Note.—The product used in this experiment was a mixture of still aggregated extracts from several lots of bacteria (CO_2-sensitive).

Huddleson (1943) reported moderate protection of guinea-pigs by means of an extract obtained by passing *Br. abortus* through a wet bacterial mill. The extract was extremely unstable, its immunizing capacity being destroyed by 56° C. or 0·5 per cent phenol, and it seems unlikely therefore that the active constituent is related to the material we find effective. Protection of guinea-pigs with an extract was also reported by Live, Sperling and Stubbs (1945) using a sonic method, which is believed not to denature labile somatic antigens (Chambers and Flosdorf, 1936). They describe their product as containing protein. There is reason to think that it may contain material resembling the antigen described by us.

In our preliminary experiments on iummnization, fractions which had been disaggregated by exposure to phenol or pyridine did not induce such a high degree of resistance as those which were still in the aggregated state. Our observations on the skin reactions in sensitized animals, on the other hand, showed that the largest swellings were provoked by the disaggregated products. It would appear, therefore, that there are different optimal particle sizes for skin testing and immunization. It is also clear that an intracutaneous method for the detection of sensitized cattle might be elaborated. It would be of practical value only if the skin reaction can be elicited by material so far disaggregated that it no longer leads to the development of agglutinins in normal animals.

Having shown in the earlier experiments that particle size was of importance, and having tested products prepared in a variety of ways, but all of which were thought likely to have good immunizing properties (Tables I, II and III), attention was next directed to mode of administration and dosage. The finding (Tables IV and V) that division of the inoculum into two injections with an interval between was better than increase of the size of a single dose was not unexpected. Further division (Table V) appears of no advantage.

The variation found in successive experiments with products thought likely to have similar protective properties was often greater than expected. There is no doubt, however, that the fractions prepared have, taken as a whole, considerable protective properties. This is shown not only by the figures for positive and negative post-mortem findings recorded here, but also by the less severe infection and the marked reduction in the numbers of organisms recovered in spleen counts from vaccinated animals as compared with the unvaccinated controls.

The degree of protection is best expressed by comparison with other well-known immunizing agents, such as the American Strain 19, now in general use in the U.S.A. and Great Britain. It is now well established (Weybridge results, unpublished) that living vaccines prepared with S. 19, which confer a high resistance on cattle, fail to protect approximately 15, 20 and 50 per cent of guinea-pigs respectively against the three test doses generally used in the work reported here (i.e. except in the experiment in Table I). de Ropp (1945) has already published somewhat similar results using the same methods. Comparison of the results obtained with our fractions shows that the level of protection was sometimes similar to, or once (with two doses), greater than that conferred by S. 19, but that with single doses it was usually definitely lower.

The relative values of antigens prepared from CO_2-sensitive and aerobic strains have not been adequately investigated, but such evidence as we have does not indicate that the former are greatly superior.

Such questions as the duration of immunity and the possibility of combining

the antigen with living (avirulent) vaccine have not yet been considered, and further work is needed on particle size, but we feel that the evidence that has been accumulated regarding these antigens should be presented at this stage.

SUMMARY.

The methods employed to isolate an antigenic substance from suspensions of *Br. abortus* are described together with its physical and chemical properties.

The inoculation of the antigen by various routes, in doses as low as 0·0003 mg., into normal animals causes the production of circulating agglutinins. When large doses are inoculated intradermally symptoms of toxaemia develop in addition to massive local necrosis at the site of inoculation.

The intradermal inoculation of small doses of antigen into sensitized animals is marked by local oedema and swellings which tend to persist for lengthy periods.

As judged by guinea-pig tests, the antigen possesses considerable immunizing powers. The resistance conferred by a single dose of 0·3 mg., or by larger doses, was however usually lower than that produced by one dose of living *Br. abortus* of low virulence, e.g. S. 19. The results with two doses of 0·3 mg. or with four doses of 0·15 mg. varied, but more nearly approached those obtained with strain 19.

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